IDENTIFICATION OF HUMAN PAPILLOMA VIRUS TYPE 16 AND 18 USING TYPE SPECIFIC PRIMERS IN NORMAL ORAL MUCOSA, EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA BY POLYMERASE CHAIN REACTION

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CERTIFICATE

This is to certify that this dissertation titled **"IDENTIFICATION OF HUMAN PAPILLOMA VIRUS TYPE 16 AND 18 USING TYPE SPECIFIC PRIMERS IN NORMAL ORAL MUCOSA, EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA BY POLYMERASE CHAIN REACTION"** is a bonafide dissertation performed by **Dr. Raghu Dhanapal**, under our guidance during his post graduate period 2004 - 2007.

This dissertation is submitted to THE TAMILNADU DR. M. G. R. MEDICAL UNIVERSITY, in partial fulfillment for the degree of MASTER OF DENTAL **SURGERY** in **ORAL AND MAXILLOFACIAL PATHOLOGY, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

RAGAS DENTAL COLLEGE * Chennai

Dr. K. Ranganathan, MDS, MS (Ohio)
Professor& HOD
Department of Oral and Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. K. RANGANATHAN, MDS, MS (OHIO)
Professor and Head of Department
Oral and Maxillofacial Pathology
RAGAS DENTAL COLLEGE & HOSPITAL
Chennai - 600 119.

Dr. M. Uma Devi, MDS
Professor
Department of Oral and Maxillofacial Pathology
Ragas Dental College & Hospital
Chennai.

Dr. M. UMADEVI KRISHNAMOHAN RAO, MDS
Professor Oral and Maxillofacial Pathology
RAGAS DENTAL COLLEGE & HOSPITAL
Chennai - 600 119.

Dr. S. Ramachandran, MDS
Principal
Ragas Dental College & Hospital
Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE & HOSPITAL
CHENNAI

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Introduction

Cancer of the oral cavity is a major cause of morbidity and mortality worldwide. Among all cancers, oral cancer ranks sixth in the developed countries and third in India[39]. Exposure to risk factors such as tobacco smoking and chewing, alcohol and viral agents, increases the risk of occurrence of cancer. The role of certain viruses in oncogenesis has been established, and its study is an important source for the understanding of the molecular basis of multistep carcinogenesis.

Viruses are derived from the Latin word "slime" or "poisonous juice". Viruses were not understood as unique entities until the 19th century [25]. Viruses have the ability to alter the growth and morphology of the cell they infect. Human papilloma virus (HPV) has been shown to contribute to cervical and oral carcinogenesis. HPV infection is initially asymptomatic (latent) and transmission can occur between people before overt expression of the virus is seen [95].

Papilloma viruses are small, non-enveloped viruses. They are double stranded DNA viruses with a circular DNA genome of about 8kb molecular weight. The viruses evade the immune system by evolution of viral variants. There are more than 100 types of HPVs. In the oral cavity at least 36 HPV types are found [18]. The mucosal HPV types are commonly grouped into “high-risk” and “low-risk” categories on the basis of known epidemiologic associations with malignancy. High-risk types are frequently found in anogenital malignancies. Low-risk types are found in benign lesions such as warts [19].

Maintenance of viral genome in the infected cells causes persistent infection. HPV infection of the dividing basal epithelial cells leads to a replication of the viral genome. These synthesize certain oncoproteins that have the potential to cause malignant transformation of the epithelial cells [59].

Studying the natural history and epidemiology of HPV is complex because of the presence of many subtypes. It is now known that HPV types 16 and 18 are carcinogenic to humans. Identification of HPV relies on the detection of viral DNA. HPV genome consists of three types of genes based on their function. They are early genes, late genes and long control region. Early and late genes produce early and late proteins. The long control region contains promoters and enhancers for the viral gene transcription.

Given the fact that all HPV types are closely related, assays can be designed to target conserved regions of the genome or regions whose sequences can best be used to discriminate different HPV types. Polymerase chain reaction (PCR) can be used to identify HPV DNA, as it is a sensitive technique for detection of even minimal quantities of viral DNA.

Different samples, like liquid nitrogen frozen tissue, paraffin embedded tissue; exfoliated oral cells in saliva can act as a source of DNA for the identification of HPV DNA[58, 46, 22]. In case of samples processed from tissues originally fixed in formaldehyde and embedded in paraffin, the smaller the PCR amplification product the higher is the rate of HPV DNA detection[3].

The PCR technique can detect and amplify even small fragments of DNA or RNA. The automated machine helps in the analysis of a large number of samples. The PCR technique, can in ideal conditions, detect even one copy of viral DNA and therefore it is highly sensitive. The present study was undertaken to identify the presence of HPV in oral squamous cell carcinoma (OSCC), epithelial dysplasia and normal mucosa by PCR technique.

Aims & Objectives

AIMS AND OBJECTIVES

1. To ascertain the presence of HPV 16 and 18 in sections from formalin fixed paraffin embedded tissue of normal oral mucosa by polymerase chain reaction (PCR).
2. To ascertain the presence of HPV 16 and 18 in sections from formalin fixed paraffin embedded tissue of epithelial dysplasia by PCR.
3. To ascertain the presence of HPV 16 and 18 in sections from formalin fixed paraffin embedded tissue of oral squamous cell carcinoma (OSCC) by PCR.

HYPOTHESIS

1. HPV 16 and 18 are present in epithelial dysplasia.
2. HPV 16 and 18 are present in oral squamous cell carcinoma.
3. HPV 16 and 18 are absent in normal mucosa

Materials & Methods

Study Design

A retrospective study was done to identify HPV DNA type 16 and 18 in oral squamous cell carcinoma (OSCC), epithelial dysplasia and normal oral mucosa.

Study Groups

Group I comprised of cases with oral squamous cell carcinoma

Group II comprised of cases with epithelial dysplasia

Group III comprised of cases with normal oral mucosa

Study Setting

The study was conducted in the Department of Oral and Maxillofacial Pathology at Ragas Dental College in collaboration with Department of Molecular Reproduction Development and Genetics, Indian Institute of Science, Bangalore.

Tissue Specimens

- Tissue specimens were retrieved from the archives of 35 histopathologically confirmed OSCC cases, from January 2004 to December 2005. The amplification of the housekeeping gene was positive for 14 cases of the 35 cases. The final sample in Group I was thus limited to 14 cases.
- Tissue specimens were retrieved from the archives of 15 histopathologically confirmed epithelial dysplasia cases, from January 2004 to December 2005. The amplification of the housekeeping gene was positive for 5 cases of the 15 cases. The final sample in Group II was thus limited to 5 cases.

- 10 normal oral mucosa were biopsied from cases, with no habit of smoking, alcohol consumption, chewing or any systemic disorders. In these 10 cases the amplification of the housekeeping gene was positive for 4 cases. The final sample in Group III was thus limited to 4 cases.

Patient profile, which included the age, gender, habit, site of the lesion and histopathology were documented for Group I, Group II (from the patient records) and for Group III, also the demographic characters were recorded. Informed consent was obtained for all patients from whom biopsy was obtained.

Basic Armamentarium

1. Microcentrifuge tubes
2. Sterile tips
3. Gloves
4. Vials 0.2 / 0.5 µl
5. Micropipettes 2 µl, 20µl, 200µl, 1000µl

Armamentarium for DNA Extraction

1. Xylene
2. Ethanol
3. Qiagen MiniAMP DNA extraction kit
4. Thermostat (Eppendorf Thermostat Plus)
5. Centrifuge (Sanyo Micro Centuar)

Armamentarium for DNA Amplification

1. Thermocycler (GENEAMP PCR system 9700) AB Biosystems

2. Master mix (FINNZYMES – DyNAzyme II)
    i. 200 µl Deoxynucleotide Triphosphatase (dNTPs)
    ii.1.5 mM Magnesium Chloride (Mgcl)
    iii. 2.5 U Taq-polymerase
3. HPV Primers
    i. HPV 18 E1 primers
        Forward Primer 5' CATTTTGTGAACAGGCAGAGC
        Reverse Primer 5' ACTTGTGCATCATTGTGGACC
    ii. HPV 16 E7 primers
        Forward Primer 5' AGCTCAGAGGAGGAGGATGAA
        Reverse Primer 5' GGTTACAATATTGTAATGGGC
4. House Keeping gene primers
    i. Glutamate Dehydrogenase Primers
        Forward Primer 5' CTGGCTTGGCATACACAATG
        Reverse Primer 5' GCTGTTCTCAGGTCCAATCC

Gel electrophoresis

1. Agarose gel, Ethidium Bromide
2. Parapin paper
3. Bromophenol Blue Dye
4. DNA Ladder
5. Gel electrophoresis tank
6. Voltmeter
7. Gel Dock (Kodak Image Station 440)

Deparaffinisation of tissue sections

The tissue sections were collected in the microcentrifuge tube; 40µm thick paraffin embedded section was used. Care was taken to prevent contamination during tissue sectioning. New blades were used for each block. After sectioning for one block the area was cleaned with xylene.

1. 1200µl of xylene was added, pulse vortexed and centrifuged at full speed. The supernatant alone was removed. Caution was taken not to remove the pellet.
2. 1200µl of 100% ethanol was added to remove xylene, pulse vortexed at slow speed and centrifuged at full speed. The supernatant alone was removed caution was taken not to remove the pellet.
3. Ethanol from the pellet is removed completely by incubating the open microcentrifuge tube at 37 degree for 15 mins.

DNA extraction

1. 180µl Buffer ATL (composition not revealed by Qiagen) and 20µl of Proteinase K was added to the deparaffinised sample in the microcentrifuge tube and was incubated overnight at 56^{o}C.
2. 200µl Buffer AL was added into the microcentrifuge tube and mixed by pulse-vortexing for 15 sec and heated at 70 ^{o}C for 10 mins.
3. The entire lysate was mixed with 200µl of 100% ethanol and was transferred to the silica based spin column with a collection tube and centrifuged (8000 rpm) for 1 min.
4. The collection tube containing the flow-through filtrate was discarded.

5. After washing with wash buffers (AW1 and AW2), the spin column was eluted with 20µl of elution buffer and the filtrate of the samples collected in a new microcentrifuge tube was stored at $-20^{o}C$.
6. The presence of DNA was checked on a 1% agarose gel (*Figure:* 9) and with a spectrometer at 260 nm.

PCR AMPLIFICATION

1. 25µl of the PCR master mix along with primer and autoclaved water was added for a final 50µl reaction. The cocktail of all these products were aliquoted into separate reaction tubes.
2. Template was added separately into the different reaction tubes in a different room to avoid contamination.
3. The final 50µl product was amplified in a Thermocycler.

PCR Amplification of house keeping gene

A fragment of the human Glutamate Dehydrogenase (Glu DH) gene was amplified with Glu DH primers.

A positive Glutamate Dehydrogenase amplification proved that the sample contains enough DNA and that no PCR inhibitors were present. If the result of the house keeping gene amplification is negative the sample was not used.

50µl of the reaction mixture should contain 1x concentrated master mix, 1.5mmol $MgCl_2$, 200 mmol dNTPs, 10 nanogram per lamda of each primer.

Calculation

Master mix comes in 2x concentration (conc.) to make it 1x conc.

$$N_1V_1 = N_2V_2$$

For 50 µl Reaction: V_2 = 25 µl of master mix for 1x reaction

Cocktail preparation for one sample

1x master mix 25 x 1 sample = 25 µl

Forward Primer 1 x 1 sample = 1µl

Reverse Primer 1 x 1 sample = 1µl

DNA Template 3 x 1 sample = 3µl

Autoclaved water is added to make up the remaining volume for 50 µl

[50 – (25+1+1+3) =20µl]

After an initial denaturation for 5 min at 94^{o}C, each of the 35 cycles will denaturate for 1 min at 94^{o}C, primer annealing for 2 min at 65^{o}C, and chain elongation for 2 min at 72^{o}C. The last cycle will be followed by the incubation for 3 min at 72^{o}C.

PCR Amplification HPV genes

Samples with positive amplification of the control gene was subjected to HPV DNA detection with HPV 18 E1 gene type specific primers

Forward Primer 5' CATTTTGTGAACAGGCAGAGC

Reverse Primer 5' ACTTGTGCATCATTGTGGACC

This generated a 73 -bp long fragment of the E1 gene.

Also detection of HPV 16 E7 gene type specific primers

Forward Primer 5' AGCTCAGAGGAGGAGGATGAA

Reverse Primer 5' GGTTACAATATTGTAATGGGC

This generated a 75 -bp long fragment of the E7 gene.

Calculation

Master mix comes in 2x conc. to make it 1x conc.

$$N_1V_1 = N_2V_2$$

For 50 µl Reaction: V_2 = 25 µl of master mix for 1x reaction

Cocktail preparation for one sample

1x master mix 25 x 1 sample = 25 µl

Forward Primer 1 x 1 sample = 1µl

Reverse Primer 1 x 1 sample = 1µl

DNA Template 3 x 1 sample = 3µl

Autoclaved water is added to make up the remaining volume for 50 µl

[50 – (25+1+1+3) =20µl]

After an initial denaturation for 3 min at 95^{o}C, each of the 35 cycles will consist of 30 seconds of denaturation at 95^{o}C, primer annealing for 30 seconds at 55^{o}C, and chain elongation for 30 seconds at 72^{o}C. At the end an extra incubation for 5 min at 72^{o}C was carried out.

Sterile water, DNA from SiHa, (a human cervical cancer cell line containing one or two copies of the HPV 16 genome per cell) and DNA from HeLa, (a human cervical cancer cell line containing ten to fifty copies of the HPV 18 genome per cell) will be included in every run as a positive control and negative control.

The PCR products were electrophoresised on a 3% agarose gel, stained with ethidium bromide, visualised under ultraviolet light, and photographed. Samples that are positive by PCR revealed a clear band on the agarose gel (3%).

Statistical Analysis

Data entry and statistical analysis was performed with the aid of Statistical Package for Social Science software version-10.0.05 (SPSS version 10.0.05). A descriptive analysis was presented for all variables.

Pearson Chi-square test of association was used to find the association of HPV type 16 and 18 in different histological grades of oral squamous cell carcinoma (Group I), epithelial dysplasia (Group II), normal buccal mucosa (Group III), and to compare the presence of HPV between the above three groups. $p < 0.05$ was considered statistically significant.

Review of Literature

Papilloma virus

History

Shope and Hurst (1933)[73] discovered the papilloma virus in cottontail rabbit as the first DNA virus to cause tumor in animals. It causes benign cutaneous papilloma in cottontail rabbit.

Strauss MJ, Shaw EW, Bunting H et al (1949)[76] showed the presence of viral particles by electron microscopy and also detected the presence of intranuclear inclusion bodies, in human warts.

Meisels A and Fortin R (1976)[53] were the first to study the role of papillomavirus infections in cervical smears and mild dysplastic lesions. The author stated that the presence of koilocytes in the smears indicate the presence of papillomavirus.

Gissmann and zur Hausen (1977)[29] identified and characterized different types of HPV by the use of molecular cloning. They also identified HPV types 6 and 11 in laryngeal warts.

Durst M, Gissmann L, Ikenberg H (1983)[20] identified the presence of HPV in cervical cancer tissue. The subtype HPV 16 was isolated from cervical cancer.

Syrjanen K, Syrjanen S, Lamberg M (1983)[81] showed the morphological and histopathological evidence of Human papilloma virus (HPV) in OSCC. They speculated the role of HPVs in the etiology of oral squamous cell carcinoma.

Von Knebel DM, Oltersdorf T, Schwarz E (1992)[90] based on their observation stated that E6 and E7 genes are necessary factors for the malignant

phenotype of HPV positive cervical cancer cell lines and also for malignant transformation of nude mice cells.

International Agency for Cancer Research Monograph (1995)[35] stated that HPV16 and HPV18 were the causative agents for cancer of the cervix. Genital low risk HPVs are the etiological agents for condyloma, recurrent respiratory papillomas and papilloma at other mucosal surfaces. It was also established that transmission of the genital warts was through sexual contact. Transmission of all HPV types is more in the presence of an abraded epithelial surface.

Miller CS, White DK (1996)[56] reviewed the literature for the identification of the expression of HPV in oral mucosa, oral premalignant lesions and normal mucosa. He identified increasing frequency of HPV infection in normal oral mucosa than cancer cases.

Pathogenesis

Receptors of HPV

Shafti-Keramat S, Handisurya A, Ernst K et al **(2003)**[70] used two cell lines, one without heparan sulphate proteoglycan (HSPG) and other cell line with syndecan-1, syndecan-4, or glypican-1 and found that the binding of the virus like particle (VLP) was dependent on the presence of HSPG. Based on these results, the authors concluded that several HSPGs can serve as HPV receptor and that HSPGs is the primary receptor proteins for natural HPV infection of keratinocytes.

Genther Williams SM, Disbrow GL, Schlegel R et al (2005)[28] used transgenic mice in which the E5 (Early) gene of the HPV16 was inserted into the Keratin 14 (K14) promoter. K14 is found in the basal layer of the stratified squamous epithelium, the site of papilloma viral infection. Epidermis of transgenic mice was used for immunohistochemical staining by bromodeoxyuridine (BrdUrd), which helps to determine DNA synthesis. Immunofluorescence staining for K14, keratin 10 (K10), and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was used for assessing epithelial hyperplasia. Western blot analysis was done to detect epidermal growth factor receptor (EGFR) after injecting epidermal growth factor to the mice .The mice with highest expression levels of E5 displayed the highest percentage of suprabasal DNA synthesis. Based on the phenotype and mutational assay the authors stated that requirement of EGFR is required for hyperplasia which is induced by E5 oncoprotein.

Cell Entry

Bousarghin L, Touze A, Sizaret PY et al (2003)[6] the authors used drugs acting on the endocytosis, to study the entry of virions. They used virus like particle (VLP) and mediated pseudoinfection, to study the entry of these virions. VLP internalization was studied by electron microscopy. The endocytosis can be of two types clathrin and caveola mediated. The inhibitors of clathrin-coated vesicles (chlorpromazine) and caveola vesicles (nystatin) were used to study virion entry. HPV-16 and HPV-58 VLPs which used clathrin-coated vesicles, appeared as smooth grapes on the plasma membrane when examined by electron microscope. HPV-31 VLPs showed the presence of noncoated flask-shaped invaginations of the plasma membrane which had the characteristics of caveola

structures. Different types of HPV used different types of endocytosis to enter the cell.

Yang R, Yutzy WH, Viscidi RP et al (2003)[92] examined the transport of the virus like particles (VLP) with capsid proteins viz L1 and L2 inside the cell by means of confocal microscope. The microfilament actin is polymerised with the VLP, in the presence of the capsid proteins. The polymerization causes the particles to co-localize with the actin microfilaments and thus helps in the transport of the particles within the cell.

Nuclear entry

Day PM, Baker CC, Lowy DR, Schiller JT et al **(2004)**[16] unraveled the HPV entry into nucleus. After the virus enters into the nucleus, finding a location in a preexisting transcription environment stimulates initial viral transcription. By using pseudogenome labeled with BrdUrd and tagged L2 protein, they showed that Papilloma viral L2 protein which mediates the delivery of the viral genome into the nucleus occurs at the Nuclear Domain 10 (ND 10) region.

Viral Capsule

Okun MM, Day PM, Greenstone HL et al (2001)[63] examined the need for interaction between the capsid proteins L1 and L2 for encapsulation. *In vitro* study using bovine papilloma virus (BPV) L1 protein with BPV L2 protein led to encapsulation. BPV L1 with HPV L2 protein did not form a capsule, but chimeric (fusion proteins) of HPV L1 and BPV L2 protein led to encapsulation. L1-binding to L2 is required prior to completion of capsid assembly was demonstrated by this experiment.

Viral Proteins

Early 1 (E1) Protein

Swindle CS, Engler JA et al (1998)[80] showed that viral E1 protein is a DNA helicase responsible for initiation of DNA replication. E1 protein is recruited to the viral origin (starting site of replication), which is facilitated by binding to E2, for which specific recognition elements are located on the virus. The remaining replication functions for the virus, is provided by the host cell's replication machinery. The site of binding of the viral E1 protein to the host replicatory site was the histone protein 1(H1). H1 was identified as E1-binding protein by Western blotting in HPV type 11 (HPV-11). HPV origin facilitates initiation of DNA replication by exclusion of H1.

Liu JS, Kuo SR, Makhov AM et al (1998)[44] Human chaperon proteins (Hsp70 and Hsp40) independently and additively enhanced E1 binding to the site of replication as shown by electrophoretic mobility shift assays. This binding also leads to initiation of E1 mediated DNA replication.

Maido R, Anu R, and Mart U et al (1999)[48] proved that E1 protein is expressed from polycistronic mRNA containing E6, E7, and E1 open reading frames (ORFs). The translation of adjacent E7 and E1 ORFs is independent and is performed by separate populations of ribosomes. Ribosome scanning precedes the translation of the downstream E1 gene and the scanning happens at the 5' end of the polycistronic mRNA as shown by vector studies. They also established that the papillomavirus expression of replication protein E1 depends on a promoter in the flanking region or a specific mechanism that would ensure the expression of the E1 protein from the multicistronic messenger.

Ma T, Zou N, Lin BY, Chow LT et al (1999)[47] did an *in-vitro* study using insect cell with plasmids of E1, E2, cyclin dependent kinase (Cdk) and cyclin E for protein interaction assay. HPV replication was studied by E1 expression plasmids. Sequence analysis of cDNAs revealed several known Cdk-interacting proteins, but E1 protein was the most abundant Cdk-interacting protein. E1 and E1/E2 were found to be associated with Cyclin E, independent of Cdk. It was also shown that ectopic expression of cyclin E could initiate replication independent of Rb inactivation in mammalian cells. Interaction of cyclin/Cdk complexes with E1 is critical for efficient replication, because cyclin/Cdk complex during HPV replication leads to phosphorylation of E1.

Early 2 (E2) protein

Masterson PJ, Stanley MA, Lewis AP et al (1998)[52] by deletion mutant assay showed that E1 carboxy terminal was necessary for binding of E2 viral protein. E1 carboxy terminal also helped in binding to the viral origin of replication (ori). The use of antibodies blocked the DNA polymerase binding to E1, but not the E2 or ori binding. This was because the host DNA polymerase binds to a different site on E1 protein.

Susanne IW, Delicia AF, Alla YK et al (2000)[79] studied the effect of E2 protein on HPV 18 positive cancer cell lines (HeLa). E2 viral protein was transfected along with the oncoproteins E6 and E7.Transfection with the full-length E2 protein only resulted in a dramatic inhibition of cell colony formation on the medium (soft agar). E6 and E7 when coinfected with E2 resulted in the formation of cell colonies. Based on these results it is shown that HPV E2 induces cellular senescence in HPV-positive cervical cancer cells and viral oncoproteins rescue HeLa cells from E2-induced senescence.

E5 protein

Chen S L, Lin S T, Tsai T C et al (2006)[12] studied the effect of HPV E5 protein and its interaction with Erb-4 (tyrosine kinase receptor) by transfection of the cells. They examined the binding of HPV E5 protein to ErbB4, by using 10 different ErbB4 deletion mutants. They were also able to show that HPV E5 protein inhibited the c-Jun protein expression and phosphorylation, resulting in increased cell proliferation. The transfected cells were serum starved for 24 hrs, followed by treatment with Epidermal Growth Factor. Cell lysates were then analysed for c-jun antibody.

Early 6 (E6) Oncoprotein

Kiyono T, Hiraiwa A, Fujita M et al (1997)[41] in their study showed the effect of E6 oncoprotein on tumor suppressor protein, human homologue of the Drosophila discs large tumor suppressor (Dlg). Binding of viral oncoprotein E6 to the Dlg protein was due to the presence of a PDZ domain (P-post-synaptic density protein, D-Dlg protein and Z-zonula occludens 1 protein) in the E6-binding protein. The E6 protein of high-risk mucosotropic HPVs also has the similar conserved amino acid motif (part of aminoacid). This interaction causes degradation of Drosophila discs large tumor suppressor protein which in turn induces E6-dependent transformation of rodent cells.

Patel D, Huang SM, Baglia LA, McCance DJ et al (1999)[64] studied the effect of E6 oncoprotein on co-activators CBP and p300, which are required for cell differentiation and cell cycle progression. The binding of E6 oncoprotein binding to CBP and p300 caused an inhibition of the transcriptional activity of the co-activators. The authors demonstrated this inhibition of the transcriptional activity by an *in-vitro* experiment using promoter elements of various cellular

proteins that are transcriptional activated by co-activators of CBP and p300. E6 could disrupt the binding of c-Fos to CBP .The activation of p53 and NF-κB by p300 is inhibited by E6 binding.

Veldman T, Liu X, Yuan H, Schlegel R et al (2003)[89] studied the functional role for Myc protein in E6-mediated activation of the human telomerase catalytic subunit gene's (hTERT) promoter. In reporter assays in Human Foreskin Keratinocyte cells, Mad reduced E6-mediated activation of the hTERT promoter. These authors showed that this decrease in promoter activity by Mad was the result of a specific repression on endogenous Myc activity, because overexpression of Myc restored E6 function to activate the hTERT promoter. Myc-binding site was necessary for E6-induced activation of the hTERT promoter. Based on this study it is concluded that E6 and Myc interact functionally to induce hTERT gene expression. The telomerase over expression in turn leads to cellular immortalisation.

Stewart D, Ghosh A, Matlashewski G et al (2005)[75] studied the coexpression of both p53 and high-risk HPV type 18 E6 (18E6) and also the shuttle for p53 from the nucleus to the cytoplasm. Inhibition of the nuclear export of p53 resulted in the partial degradation. The study showed that p53 in the cytoplasm was not targeted for degradation by 18E6 *in vivo* but could be degraded *in vitro*.

Early 7 (E7) Oncoprotein

Jones DL, Alani RM, Munger K (1997)[40] used an *in-vitro* study for evaluating the differentiation of human foreskin keratinocytes cells (HFK) and E7 infected HFK. From the cultured cells they studied epithelial differentiation

by means using differentiation markers involucrin and keratin 10. In HPV-16 transfected keratinocyte that expresses E7 protein only after 72 hrs of induction, the differentiation markers were observed. They also determined the levels of $p21^{Cip1}$ (Cdk inhibitor), which was considerably higher in HPV-16 E7-expressing keratinocytes compared with matched control cells at time intervals of 24 hrs and 72 hrs after induction of differentiation. Also, high levels of $p21^{Cip1}$ associated with cdk2 were detected in E7-expressing keratinocytes at 24 hrs and 72 hrs after keratinocyte differentiation. This shows that E7 interaction with p21 can cause an inhibition of p21 inhibitory activity on cyclins A and E and leads to DNA synthesis.

Martin LG, Demers GW, Galloway et al (1998)[51] studied the effect of E7 protein on different types of cyclins and Cyclin Dependent Kinase (Cdk) in human foreskin keratinocyte (HFK). HFK cells in the G1/S phase of cell cycle as determined by fluorescence assisted cell sorting (FACS). There was an increased expression of cyclin E as detected by IHC. This increased expression of cyclin E, based on the prediction that the disruption of the Rb-E2F pathway will lead to increased transcription of E2F-responsive genes. They found that there was no increase in the other E2F-responsive genes like cyclin A and cdc2. Based on this observation the authors concluded that the ability of the E7 to regulate cyclin E involves activities in addition to the release of E2F.

Flores ER, Allen-Hoffmann BL, Lee D et al (2000)[27] studied the immortalized human foreskin keratinocyte cell genome which existed in episomal form by transfecting it with the E7 deficient viral DNA. Episomal form is the state of the genome that is found in normal infections. The cells were cultured for E7-deficient HPV-16 genome and were found to have reduced amounts of the

capsid protein L1, which is required for virus production. These cells did not stain for the markers of keratinocyte differentiation, like keratin 10, involucrin, or filaggrin. E7 thus plays an essential role in the papillomavirus life cycle.

Nguyen DX, Westbrook TF, McCance DJ et al (2002)[61] studied the effect of E7 oncoprotein on cdc25 a cell cycle regulator. Using a reporter assay it is shown by these authors that E7 can transactivate the cdc25. The keratinocyte cells were serum starved which in turn decreased cdc25A gene transcription. In the presence of E7 it resulted in increased cdc25A gene expression. Based on these observations it is suggested that cdc25A is an important mediator of E7-induced S-phase entry in carcinogenesis.

Helt AM and Galloway D (2003)[33] stated that the mechanism by which HPV E7 can inactivate Rb (Retinoblastoma) family of proteins is by a functional inactivation or by means of destruction of Rb protein. The functional inactivation is due to the inhibition of binding of Rb protein and E2F. There is inefficiency for E7 to completely inactivate Rb functionally. Therefore the accelerated destruction of the Rb of the family of proteins by means of the proteasome dependent pathway is the key factor causing altered Rb protein function.

Late (capsid) proteins

Florin L, Sapp C, Streeck RE (2002)[26] showed that capsid proteins of HPV are preassembled in the cytoplasm and translocated to the nucleus. They also showed that the capsid proteins [late protein –1 (L1) and late protein - 2 (L2)] translocated into the nucleus is independent of each other. These proteins then bind to nuclear domain 10 and then interact with each other.

Yang R, Day P, Yutzy W (2003)[91] in their *in-vitro* study showed that the late proteins (L1 and L2) were necessary for causing papillomavirus infection of

the host cell. L2 region on the virion, certain epitope was necessary for infection. They used virions with deleted regions of the epitope on the late proteins. Thereby they established that papillomavirus late proteins help to cause infection.

Transmission of HPV

International Agency for Cancer Research Monograph {IACR} (1995)[35] reported that genital HPVs are transmitted primarily through sexual contact with infected cervical, vaginal, vulvar, penile or anal epithelium. Perinatal transmission, digital and oral transfers and autoinoculation of genital HPV types have also been documented. Transmission of all HPV types is probably more efficient in the presence of an abraded epithelial surface. HPV16 and HPV18 were defined as causative agents for cancer of the cervix.

Syrjanen S, Puronen M et al (2004)[82] meta analysed the prevalence of HPV infection in children. Squamous cell papilloma has been estimated to account for about 7-8% of all oral tumors in children. PCR of oral scrapings detected 4% to 87% prevalence of HPV DNA in children. HPV DNA has been detected in 37% to 73% of nasopharyngeal aspirates. Laryngeal papillomas have a tendency to recur even after a radical surgical excision. In foreskins of newborns undergoing routine circumcision, 4% were found to contain HPV DNA. There is also evidence that transmission in utero or post-natal acquisition is possible.

Bodaghi S, Wood LV, Roby G et al (2005)[5] examined HPV DNA in frozen peripheral blood mononuclear cells (PBMCs) of HIV-infected pediatric cases and healthy blood donors. PCR was done with the PBNCs using L1 and two sets of HPV type-specific E6 and E2 primers. Eight pediatric HIV cases were

positive for HPV16 DNA (14%). Nested PCR confirmed the presence of episomal HPV16 DNA in the PBMCs, by detecting the head-tail junctions of episomal HPV16 genomes. Amplicons were sequenced and showed a correct head-tail junction. Presence of HPV DNA in prostatic tissue, sperm cells, and breast cancer raised the hypothesis that HPV DNA in PBMCs could spread and infect epithelial cells in other organs.

Benign Cutaneous Lesion

Yuan-Hong L, Xing-Hua G, Chin-Di H et al (2002)[93] in a study of two cases of Darier disease found HPV subtypes 5, 36, 38 and 8 in one case and HPV 5 and 36 in another case.

Malignant Transformation Potential - Cutaneous Disease

Majewski S, Jablonska S et al (2002)[49] reviewed the potential for malignant transformation in epidermodysplasia verruciformis (EV)) an inherited autosomal disease with heterogeneous clinical appearance. They stated that HPV types 5 & 8 commonly associated with EV are not recognized by the immune system and they induce mutation. EV HPV types are also associated with psoriasis.

Cinzia M, Pawel GF, Fabrizio G et al (2003)[13] studied EV associated with HPV infections as a risk factor for development of skin cancer and suggested that HPV 8 can act as a co – factor in the development of cutaneous squamous cell carcinomas.

Benign Genital Lesions

Brown DR, Schroeder JM, Bryan JT et al (1999)[9] found that HPV types 6 and 11 are the major etiologic agents of condylomata acuminata lesions. In immunosuppressed cases the significance of additional "high risk" HPV types 16, 55, and 59 in these lesions has not been established. Foci of high-grade dysplasia have been identified in condylomata acuminata lesions.

Terai M, Takagi M, Matsukura T et al (1999)[86] identified the presence of HPV in a case of oral viral wart based on histopathologic, immunohistochemistry and molecular studies (In-situ hybrisation and Southern blot assay).In this case they detected the presence of the subtype HPV 2.

Michael D (2005)[54] stated that people with oral cancers containing the antibodies to HPV16 strain were three times more likely to report of having had oral sex than those in whom the tumour did not contain HPV16. Cases with oral cancer were also three times more likely to have antibodies against HPV than the healthy controls.

Malignant Transformation

Ferber MJ, Thorland EC, Brink AA (2003)[24] used the RS–PCR technique to screen for HPV18 integration in a total of three HPV18-positive cervical carcinoma cell lines and 32 cases of HPV18-positive primary cervical carcinomas. Many of the HPV18 integration sites were found to occur in human repetitive sequences. Many of the integrations were also associated with deletions and/or complex rearrangements. By FISH method it was shown that the integration of HPV 18 occurred at Common Fragile sites (CFS), regions of genomic instability.

63% integration occurred in CFSs. 30% of all HPV18 integrations occurred at the chromosomal band 8q24 near the c-myc proto-oncogene.

Pradip N, Kumaravel S, and Sudhir K (2003)[66] by an *in-vitro* study analysed the relation between activated Notch1 (AcN1) and p53 function mediated through the PI3K pathway. Activation of a p53-dependent reporter showed that AcN1 reduces the induction of p53 sevenfold. *In vitro* transformation assays showed that activated forms of PKB/Akt along with AcN1 and in the presence of E6, led to transformation of cells in soft agar.

Al Moustafa AE, Foulkes WD, Benlimame N et al (2004)[1] by an *in-vitro* study examined the co-factor for HPV induced carcinogenesis in head and neck cancers. Overexpression of ErbB-2 or E6/E7 alone in normal oral epithelial cell lines does not affect E-cadherin/catenin complex pattern. There was a positive effect of the cooperation between oncoproteins E6 and E7 with ErbB-2 receptor on the E-cadherin/catenin complex patterns, on the neoplastic transformation of human normal oral epithelial (NOE) cells. This was demonstrated by formation of colonies in soft agar and neoplastic transformation in nude mice. E6/E7/ErbB-2 cooperation induced nuclear translocation of β-catenin, which in turn causes overexpression of cyclin D1 and c-myc this was determined by western blot assay. The upregulation of these proteins was suggested as a cause of neoplastic transformation.

Oishee C, Karthikeyan V, Vinay T (2004)[62] showed that the transforming potential of cell lines with a variant of E6 protein was dependent on the activation of mitogen-activated protein kinase (MAPK) signaling, which cooperates with deregulated Notch1 signaling. Transforming potential was shown

by increase in colonies in soft agar and formation of tumor in nude mice. This experiment also proved that Ras oncogene had no transforming effect on E6 variant (alteration in amino acid-83).

Schreiber K, Cannon RE, Karrison T (2004)[68] studied the synergism of HPV16 E6/E7 and v-Ha-ras *in vivo* by using bitransgenic mice (E6/E7 and v-Ha-ras) and it's relation in transformation to some HPV-associated cancers. The prevalence rate of tumors in different sites in bitransgenic mice like mouth, eye and ear was 100, 71 and 79% respectively. However, in the single transgenic mice it was shown that there existed a latency of tumorigenesis. Thus the synergistic effect of E6/E7 and ras was established by this *in-vivo* study.

Al Moustafa AE, Foulkes WD, Wong A (2004)[2] in their experimental study used normal oral epithelium (NOE) and normal embryonic fibroblast transfected the cells with E6/E7 alone and E6/E7/ErbB-2.They found that the size of soft agar colony among the cells with E6/E7 alone and E6/E7/ErbB-2 was different. In nude mice also the size of tumor was small when E6/E7 alone was used and large with E6/E7/ErbB-2. Failure to induce neoplastic transformation of cyclin D1 negative cells in the presence of E6/E7,ErbB-2 *in vivo* and *in vitro* was detected. This author concluded that cyclin D1 causes the neoplastic transformation in normal cells induced by E6/E7 or E6/E7/ErbB-2 cooperation.

Fan X, Liu Y, Chen JJ (2005)[23] investigated the mechanism of apoptosis during E6-mediated immortalization of primary human mammary epithelial cell (HMEC). Apoptosis observed in HMECs did not correlate with telomerase activity. Ectopic expression and down-regulation of the cyclin-dependent kinase inhibitor p21 $^{Waf1/Cip1}$ by E6 was determined by western blot. This decrease in p21 correlated with E6's ability to induce apoptosis.

Subramanyam D, Krishna S (2006)[77] examined transformation of immortalised human epithelium cell lines (HaCaT cells) by c-myc signalling in the presence and absence of activated Notch-1 receptor along with the presence of oncoproteins E6. The authors, transfected HaCaT cells which normally expresses E7, with either Activated Notch-1 and E6 or c-Myc and E6 or co-expression of c-myc and Notch-1, all the three groups resulted in increase in the number of colonies formed on soft agar. The results demonstrated that c-Myc could replace Activated Notch-1 in synergizing with the HPV oncogenes E6 and E7 and that the co-expression of c-Myc and Activated Notch-1 also did not affect transformation potential. Notch1 interacts with the DNA binding protein (CBF1) and causes the transcriptional regulation. This study also has shown that c-Myc can cooperate with E6/E7 in epithelial transformation and can substitute for CBF1-dependent signals generated by Notch-1.The outcome of this *in-vitro* study, identifies c-Myc as an important partner in HPV-mediated transformation of human epithelial cells.

Cervical Cancer

Burger RA, Monk BJ, Kurosaki T et al (1996)[10] studied the presence of HPV in cervical cancer and its association with the clinical behaviour. There was no difference in survival in cases with HPV.

Longworth MS, Laimins LA et al (2004)[45] reviewed the pathogenesis of HPV infection in differentiating epithelia. The oncoprotein E6 of HPV binds to p53 along with ubiquitin ligase to form a tertiary complex, which causes a reduction of p53 half life from several hours to less than twenty minutes in keratinocytes. E6, to immortalize the epithelia should activate the expression of

catalytic telomerase. Immortalisation of human keratinocyte not only requires the above mechanism but also inactivation of Rb pathway by E7 oncoprotein.

Normal Oral Mucosa

Terai M, Hashimoto K, Yoda K et al (1999)[85] detected that the prevalence of HPV in normal oral mucosa, in patients with skin warts and in patients without warts. A variation was seen in the frequency of human papillomavirus infections with a low copy numbers and also a difference was observed in the genotypes in both these oral mucosal specimens. These authors stated that the sensitivity of L1 primers is better than the type specific and general primers. Considering the fact that HPV was present in high numbers in the oral normal mucosa, the authors feel that oral mucosa is a normal reservoir for HPV.

Markette H, Jaana W, Hellevi R et al (2005)[50] in their study help to determine the source of entry for latent HPV infection in the oral cavity. Based on the fact that HPV affects the basal cells and the exposed junctional epithelium resembles basal layer the decision to study the infectious gingival pocket as source of HPV detection was taken. The authors were able to detect HPV in 26% of the gingival biopsies of cases with periodontitis, with PCR using general primers and followed by the use oligoprobes for high risk HPV. In-situ hybridisation results showed the presence of HPV in the coronal portion of the junctional epithelium.

Benign Oral Lesions

Miller CS, White DK (1996)[56] reviewed literature from 1983 to 1995 for the identification of the expression of HPV in oral mucosa, oral premalignant lesions and OSCC. They identified increasing frequency of HPV infection in

normal mucosa (13.5%) leukoplakia (14.8%) and OSCC (26.2%). HPV was detected more readily with PCR and also in samples that were fresh and frozen rather than paraffin embedded.

Miller CS, Johnstone BM (2001)[57] conducted a meta analysis of 94 articles published in Medline in English Literature to detect HPV in tissues or cells derived from normal oral mucosa, leukoplakia, carcinoma in-situ and oral cancer. The analysis was made by means of a random-effects model with and without adjustments for array sensitivity. The HPV was detected in 10% of normal oral mucosa, 22.2% of leukoplakia, 26.2% of carcinoma in situ, 29.5% of verrucous carcinoma and 46.5% of OSCC.

Jimenez C, Correnti M, Salma K et al (2001)[38] used PCR technique in biopsy specimens of normal oral mucosa and cases with squamous papilloma, oral condyloma, oral verruca vulgaris and focal epithelial hyperplasia. HPV DNA was detected in 41% of squamous papilloma, 50% of oral verruca vulgaris, 100% of oral condyloma, 100% of Heck's disease and 10% of normal mucosa. HPV 6 was the predominant type.

Chen PC, Pan CC, Kuo C et al (2002)[11] applied in situ-PCR to detect various HPV types. Their findings were as follows HPV 6 (11%), HPV 11 (3.7%), HPV 16 (85.7%) and HPV 18 (71.4%). Using multivariate analysis it showed that HPV infection and betel quid chewing were independent risk factors in contrast to the hypothesis that when risk factors such as quid and HPV infection existed together they act synergistically.

Sugiyama M, Bhawal UK, Dohmen T et al (2003)[78] used paraffin embedded sections amplified by PCR to detect the presence of HPV in normal oral mucosa, leukoplakia, OSCC (oral squamous cell carcinoma) and found that

they were 36%,61%,35% respectively. In case of oral cancer cell lines HPV was detected in the early passage of cell culture, so HPV may be involved in the early phase of carcinogenesis.

Jayasooriya P R, Abeyratne S, Ranasinghe A W et al (2004)[37] case reports of two cases with multiple whitish nodules in the oral cavity diagnosed as Focal epithelial dysplasia (Heck's disease) based on the histopathology. PCR detected HPV DNA in one case.

Head And Neck Cancers

Roberto E V M, Luisia L V, Anoi C C et al (1998)[67] determined the prevalence of HPV infection in head and neck cancers using frozen sections amplified by PCR. They found a prevalence of 11% HPV suggesting that chemical carcinogens play a more important role than HPV in cancer formation.

Mork J, Lie AK, Glattre E et al (2001)[58] in their study proposed HPV as a risk factor for head and neck cancer. They postulated that HPV DNA detection might lead to sampling errors in asymptomatic cases. They suggested that using capsid antibodies to HPV could be a reliable marker of previous and present HPV infection. The serum of 292 cases was examined and only 98 cases were positive from HPV capsid antibodies. In 228 of the biopsy specimens, HPV was detected in 160 tumor specimens. Smoking was excluded as a confounding factor using serum continine as marker. Other confounding risk factors could not be excluded. They suggested that a possible causative association between HPV and head and neck cancers could exist.

Herrero R, Castellsague X, Pawlita M et al (2003)[34] in a multi-centric, case –control study of 1670 cases from 9 countries found a prevalence of 3.9%

HPV in oral squamous cell cancers and 18.9% oropharynx cancers. They used biopsy specimens stored in frozen condition and blood samples for detection of HPV DNA. HPV type 16 was found in 89.3% of cases, HPV16 and 18 in 5.4%, only HPV18 in one case. They conclude that HPV acts as the etiologic agent in a small subgroup of oral carcinoma.

Li W, Thompson CH, O'Brien CJ et al (2003)[43] examined the expression and inactivation of genes operating within the p53 and pRb pathway, such as cyclin D1 and cyclin dependent kinase inhibitors p21, p16, p27 and also p53 and pRb proteins, in Tonsil cancer with HPV positivity / negativity and correlated it with the survival of the patient. Cell cycle proteins could not predict the recurrence and survival of the patient. Cases with HPV positive tumors were significantly less likely to have recurrence or die of the disease. Based on this they conclude HPV positive tonsil cancers are a distinct biologic group.

Gillison L (2004)[30] suggested the fact that molecular changes for HPV positive head and neck carcinoma is different from other carcinomas and hence HPV positive cancers are considered, a distinct entity. In normal oral keratinocytes when transfected with HPV 16 alone, it caused immortalization of the cells but did not form tumors in nude mice. On further exposure to tobacco it caused tumor formation.

Braakhuis BJ, Snijders PJ, Keune WJ et al (2004)[8] found that HPV positive carcinomas had no p53 gene mutation when tested by direct genomic sequencing and lower level of allelic loss when studied with microsatellite marker for 3p, 9p, 17p chromosomes. HPV positive carcinomas had genetically distinct pattern of alteration and it could be an early event in carcinogenesis.

Zhang ZY, Sdeck P, Cao J et al (2004)[94] were able to identify 55% HPV DNA subtypes 16/18 in normal mucosa and 74% in OSCC. Based on the previous studies the author feels that the use of Late gene (L) primer may give negative results, because the L1 and L2 regions may be lost during the HPV DNA integration into host DNA. The prevalence of HPV was more in young cases and predominantly in males. The authors suggested that the HPV alone is not a good predictor for progression to cancer because of its presence in normal mucosa.

Tachezy R, Klozar J and Salakova M et al *(*2005)[83] examined the prevalence and subtypes of HPV in oral and oropharyngeal cancer. HPV DNA was detected in 51.5% of cases. HPV16 was the predominant type. HPV 33 was also detected in three cases. Since there was no association with smoking or alcohol drinking the author concluded that head and neck cancer positive for HPV are a different group epidemiologically and etiologically.

Oral Cancer

Syrjanen K, Syrjanen S, Lamberg M et al (1983)[81] in their study they indicated the presence of HPV virus both morphologically and immunohistochemically. Their analysis showed morphologic alterations in superficial layer such as hyperkeratosis, dyskeratotic superficial cells, Deep layers showed acanthosis and koilocytosis, which is pathognomonic of HPV infection. Individual cells showed single cell dyskeratosis and multinucleation. They also identified immunohistochemically the presence of HPV in cancer tissue.

Miller CS, Zeuss MS, White DK et al (1994)[55] studied the prevalence of the subtypes HPV DNA in deparaffinised oral carcinoma specimens on slides

using in situ PCR. HPV 16 was detected in 69.2% of cases and HPV 18 in 28% of cases using in–situ PCR. HPV DNA was not detected in any specimens by in-situ hybridization technique. Histomorphologic analysis by in–situ PCR of HPV altered epithelium (vacuolated cells) and in areas adjacent cancer tissue, did not show positive HPV DNA.

Haraf DJ, Nodzenski E, Brachman D et al (1996)[32] examined the correlation between p53 and HPV infection in head & neck cancers, 24% of cases with p53 mutation was detected by direct genomic sequence. HPV infection was seen in 18% of 66 cases by PCR amplification. p53 mutation was seen in cases with history of smoking whereas HPV infection had an inverse relation with smoking. HPV infection was correlated with a more advanced stage of cancer and was associated with more favourable overall survival rate in stage IV cases. Thus, they concluded that p53 mutation and HPV infection occurred in different types of SCC of head & neck.

Elamin F, Steingrimsdottir H, Wanakulasuriya S et al (1998)[21] detected HPV in 14/28 cases of oral cancer and 4/12 cases premalignant lesions. Presence of HPV in premalignant lesions helped the authors to come to a hypothesis that HPV may be an early event in carcinogenesis. No association was seen between HPV and the tumor grade.

Shima K, Kobayshi I and Saito I et al (2000)[72] in their study found a significant relationship between HPV 16 of and p53 mutation. They suggest the HPV 16 has a mutagenic effect in oral OSCC. No significant relationship was observed between HPV infection and p53 mutation on survival of the patient.

Glanna B, Aldo V and Armando B et al (2000)[31] they examined cancers of head & neck they were able to detect HPV DNA by PCR in 1/3rd of cancers.

HPV was detected in 7(10) male cancer cases. High risk types HPV 16 and 18 were mostly present, but low risk types were also present and dual subtype infections were also seen. In the oral cavity HPV was seen more in tongue and the second common site was the buccal mucosa. There was no relation to tumor differentiation in HPV positive samples. Using immunohistochemical techniques for the analysis of p53 and BCL-2 over expression, p53 staining was positive in tumors cell and negative for BCL-2. This *in vivo* study showed no links of HPV infection with p53 alteration and with BCL-2 expression, in contrast to *in - vitro* studies.

Lucia G, Giuseppina C and Anna L et al (2001)[46] determined the presence of HPV DNA in different oral mucosal lesions. In squamous cell carcinoma the prevalence of HPV was higher (61.5%), 27.5% in potentially malignant lesions and 5.5% in controls. HPV type 18 was most prevalent genotype (85.7%) and HPV 16, 31 and 33 were rarely detected in this Italian study. In a different study HPV 18 was found predominantly in a genetically similar Greek population.

Seth R S, Bevan Y, James K M et al (2001)[69] studied the survival of HPV 16 positive OSCC cases (40) and HPV negative OSCC cases (214) with stage 3 or stage 4 cancer over a period of 54 months and controlling other known confounders of survival. The mean age in these HPV positive cancer cases was 54.2, males were commonly affected and tumor of the tongue was commonly HPV positive. The authors concluded that HPV 16 DNA was independent of factors and was associated with a favourable prognosis.

Kojima A, Marda H and Sugita Y et al (2002)[42] studied the correlation between HPV 38 and the expression of the proliferative cell nuclear antigen

(PCNA) and p53 in OSCC. HPV 38 was positive in 38%. Positive staining with immunohistochemistry for PCNA and p53 was higher in HPV 38 positive OSCC.

Tang X, Jia L, Ouyang J et al (2003)[84] compared the prevalence of HPV 16, 18 and 33 in Japanese and Chinese and the over expression of p53 in these cases. The prevalence of HPV 16 and 18 in both the populations was 46.7%. Over expression of p53 was more in HPV negative carcinomas. HPV 33 was not present in both the populations. A new fragment was detected on amplification of HPV 16 on genomic analysis and it was found to be the lost part of chromosome 5. HPV and loss of chromosome 5 led to cancer development and its progression.

Ibieta BR, Lizano M, Fras-Mendivil M et al (2005)[36] in their study they found that HPV is a risk factor for oral squamous cell carcinoma. HPV infection, alcohol drinking and smoking are independent risk factors. They also suggested that women with cervical cancer should be studied for the status of HPV in the oral cavity.

Results

Thirty-five cases with a confirmed histopathology of oral squamous cell carcinoma, 15 cases of epithelial dysplasia and 10 of normal oral mucosa were included for the study. DNA extraction was done for all samples. For those that were negative the first time, DNA extraction method was modified and repeated again. DNA could be retrieved only from 23 cases as ascertained by positivity for the housekeeping gene, Glutamate Dehydrogenase (Glu DH) by Polymerase Chain Reaction (PCR). This was done to rule out the possibility of PCR inhibitors and absence of DNA integrity.

Consequently the final sample was limited to *14 cases of OSCC (Group I), 5 cases with epithelial dysplasia (Group II) and 4 controls with normal oral mucosal tissues (Group III)*.

Mean age of cases in Group I was 58.4, 53 for Group II and 36.8 for Group III. In Group I there was 7(50%) males, in Group II all 5(100%) were males and in Group III 2(50%) of the cases were males. The male to female ratio was 1:1 in Group I and Group III. *(Table 1 /Graph1).*

Out of all 14 cases of Group I, 8(57.1%) were from the buccal mucosa, 1(7.1%) from alveolar mucosa, 2(14.3%) from the floor of the mouth, 1(7.1%) from gingiva, 1(7.1%) from labial mucosa and 1(7.1%) from the tongue. In Group II all of the cases were from buccal mucosa. In Group III 3(75%) were from buccal mucosa and 1(25%) from gingiva *(Table 2 /Graph 2).*

All the cases in Group I and Group II had habits of chewing and smoking tobacco and consuming alcohol. In Group I, 6(42.9%) cases had habit of chewing arecanut and tobacco, 5(35.7%) were smokers, 3(21.4%) smoked tobacco and consumed alcohol. In Group II, 2 (40%) of the cases were smokers, 1 (20%) case had

the habit of chewing and smoking cigarettes, 1(20%) had the habit of both smoking and alcohol consumption and 1(20%) was a chewer and alcoholic (*Table 3 /Graph 3).*

On histopathological examination in Group I 7(50%) was well differentiated, 6(42.9%) were moderately differentiated and 1(7.1%) was poorly differentiated grade of carcinoma. In Group II 3(60%) had histology of mild dysplasia while 2(40%) exhibited moderate dysplasia (*Table 4 /Graph 4).*

In the HPV 16 gel the positive control DNA from SiHa cell line and negative control DNA from HeLa cell line were added. Well-stained bands for SiHa were observed at 75 bp. The DNA ladder marker was added, on the last wells of each lane, with which the molecular weight was compared.

In the HPV 18 gel the positive control DNA from HeLa cell line were added and negative control DNA from SiHa cell line were added. Well-stained bands for HeLa were observed at 73 bp. The DNA ladder marker was added, on the last wells of each lane, with which the molecular weight was compared.

In the gel for house keeping gene Glu DH (Glutamate Dehydrogenase) PCR amplification the clear band was seen in 23 cases only (*Figure: 10).*

The HPV 18 was detected in 3(21.4%) cases in Group I and was not detected in Group II and Group III (*Figure: 11 /Table 5 /Graph 5)* cases. There were three bands corresponding to the 73 bp size at level below the 100 bp ladder in correspondence with the level of the band for positive control HeLa, which indicated the presence of HPV 18.

HPV 16 was not detected in Group I, Group II and Group III (*Figure: 12*). No band was detected at the 75 bp size (at a level below the last marking on 100 bp ladder) or in correspondence with the level of the band for positive controls SiHa in all three groups.

Tables & Graphs

Table 1: Distribution of Gender in the Study Groups

Groups	Male		Female		p - value
	n	%	n	%	
Group I Cancer (n = 14)	7	50	7	50	0.13
Group II Epithelial Dysplasia (n = 5)	5	100	0	0	
Group III Normal (n = 4)	2	50	2	50	
Total	14		9		

Graph 1: Distribution of Gender in the Study Groups

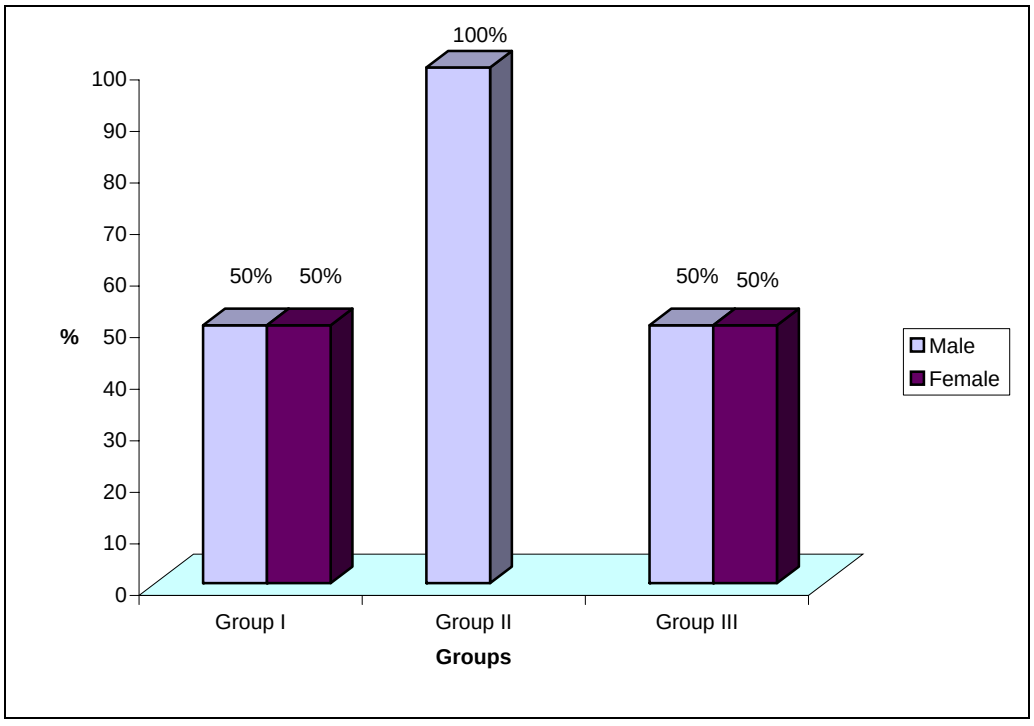


Table 2: Distribution of Site in the Study Groups

Groups	Sites												p - value
	Alveolar Mucosa		Buccal Mucosa		Floor of the mouth		Gingiva		Labial Mucosa		Tongue		
	n	%	n	%	n	%	n	%	n	%	n	%	
Group I Cancer (n = 14)	1	7.1	8	57	2	14	1	7.1	1	7.1	1	7.1	0.82
Group II Epithelial dysplasia (n = 5)	-	-	5	100	-	-	-	-	-	-	-	-	
Group III Normal (n = 4)	-	-	3	75	-	-	1	25	-	-	-	-	

Graph 2: Distribution of Site in the Study Groups

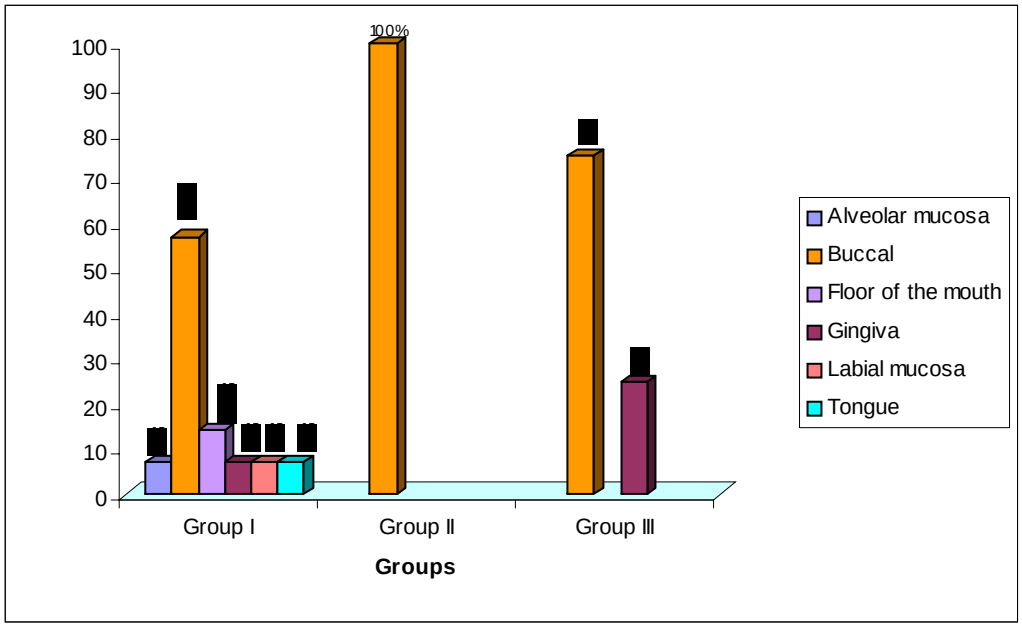


Table 3: Distribution of Habits in the Study Groups

Groups	Habit										
	Chewer		Smoker		Chewer + Smoker		Smoker + alcoholic		Chewer + alcoholic		P - value
	n	%	n	%	n	%	n	%	n	%	
Group I Cancer (n = 14)	6	42.9	5	35.7	-	0	3	21.4	-	0	0.10
Group II Epithelial dysplasia (n = 5)	-	-	2	40	1	20	1	20	1	20	
Group III Normal (n = 4)	-	-	-	-	-	-	-	-	-	-	

Graph 3: Distribution of Habits in the Study Groups

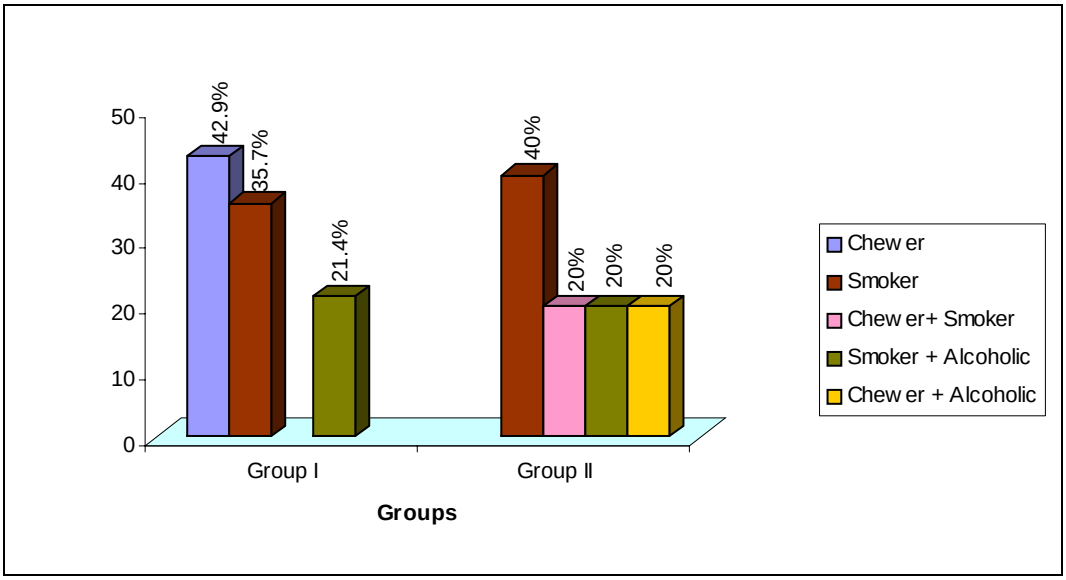


Table 4: Distribution of Histopathological Grading in the Study Groups

Group I (n = 14)						**Group II (n = 5)**			
Well differentiated		Moderate differentiated		Poor differentiated		Mild dysplasia		Moderate dysplasia	
n	%	n	%	n	%	n	%	n	%
7	50	6	42.9	1	7.1	3	60	2	40

Graph 4: The Distribution of Histopathological Grading in the Study Groups

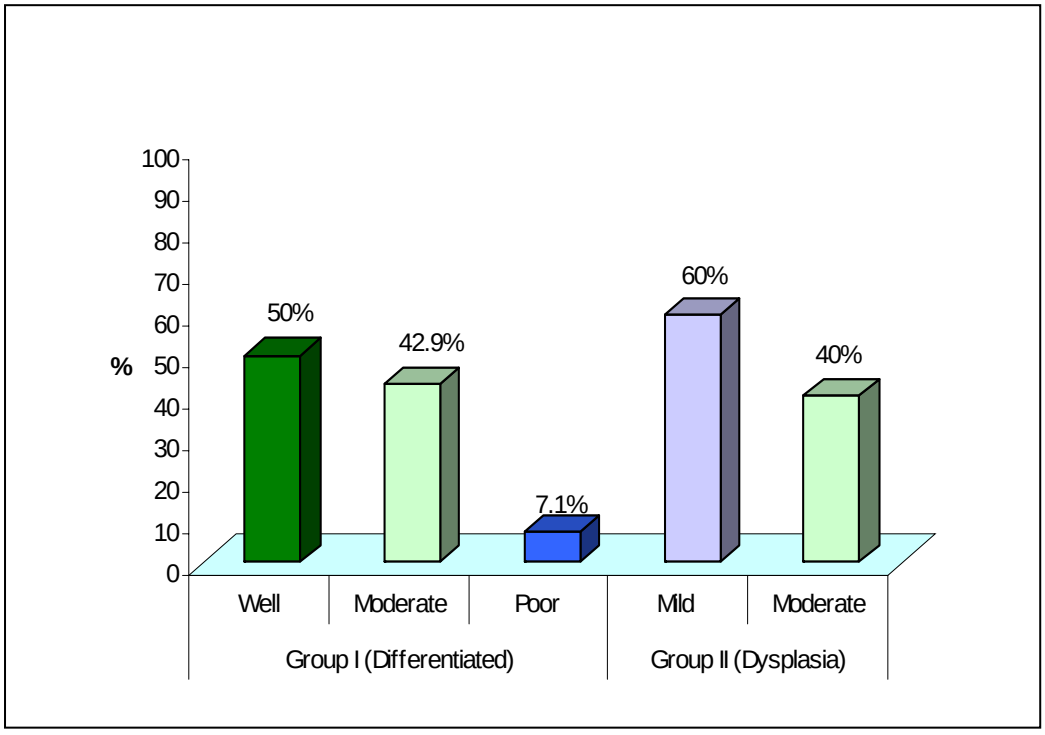
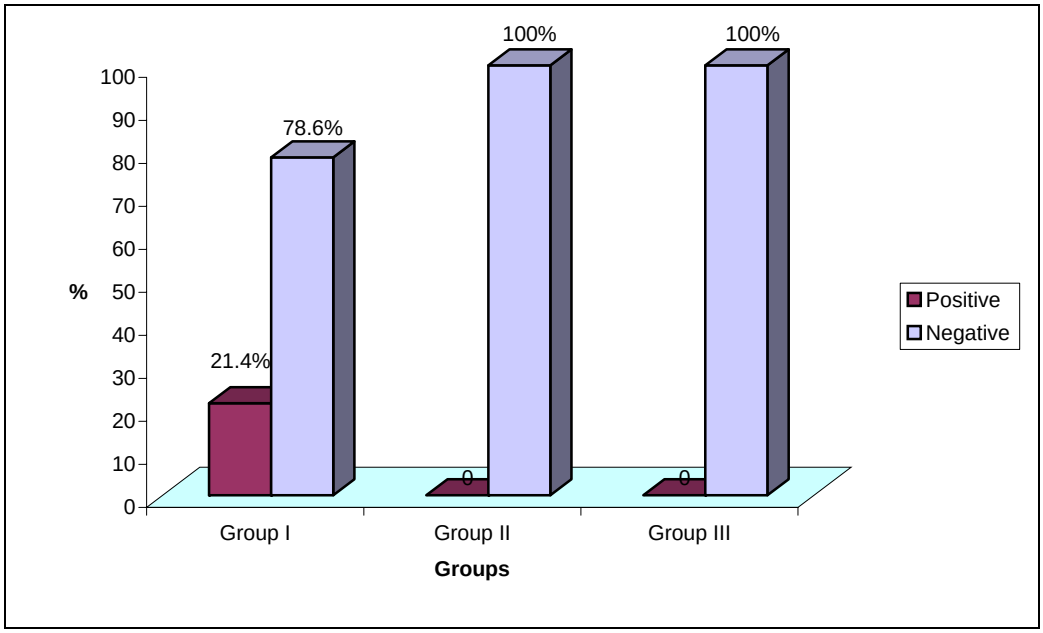


Table 5: Distribution of HPV 18 in the Study Groups

Groups	+ ve		- ve		p-value
	n	%	n	%	
Group I Cancer (n = 14)	3	21.4	11	78.6	0.33
Group II Epithelial dysplasia (n = 5)	0	0	5	100	
Group III Normal (n = 4)	0	0	4	100	

Graph 5: Distribution of HPV 18 in the Study Groups



Photographs

Armamentarium

Figure – 1: DNA Extraction Kit

Figure – 2: Pulse Vortex

Armamentarium

Figure – 3: Centrifuge

Figure – 4: Thermostat

Figure – 5: PCR Thermal Cycler

Armamentarium

Figure – 7: Gel Tray

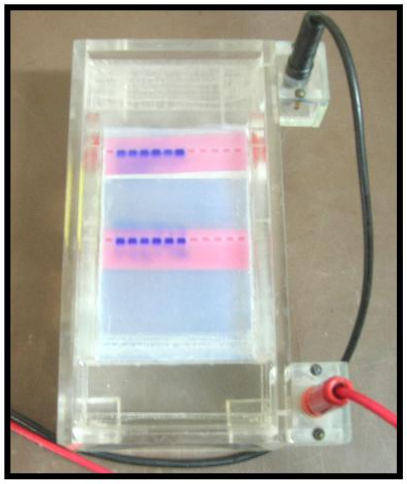


Figure – 6: Voltmeter

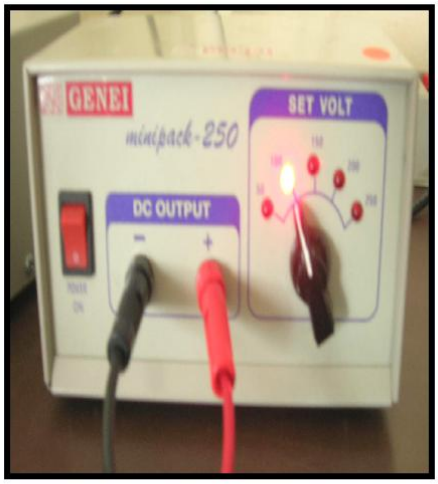


Figure – 8: Gel Dock

Gel Photographs

Figure 9: Gel showing presence of DNA in the study Groups (n = 24)

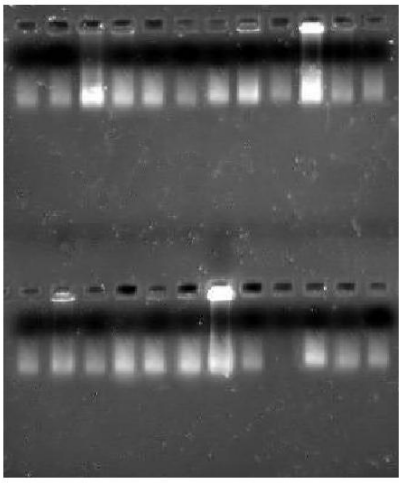
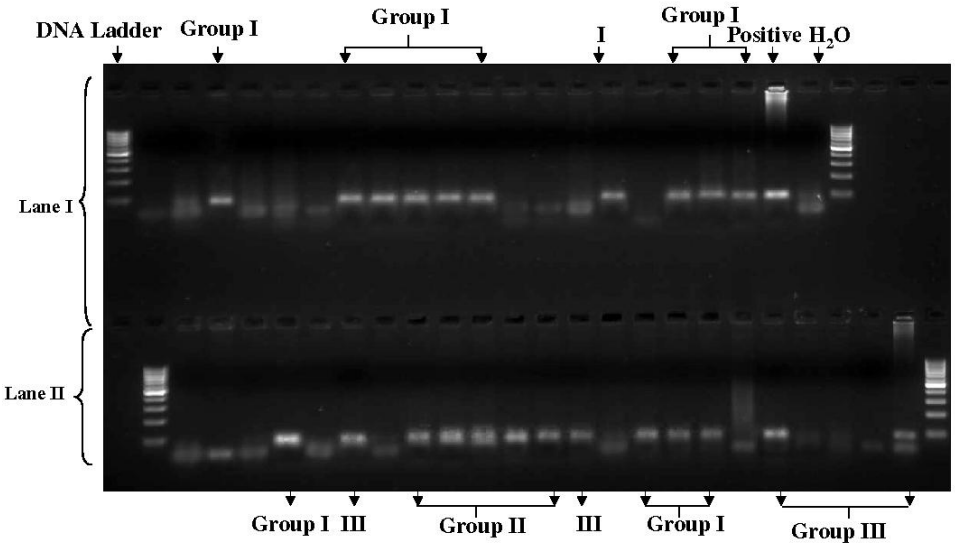


Figure 10: Gel showing Amplification of Glu DH (House Keeping Gene) **in the Study Groups** (n = 42)



Gel Photographs

Figure 11: Gel showing the Presence of HPV 18 in the Study Groups (n = 23)

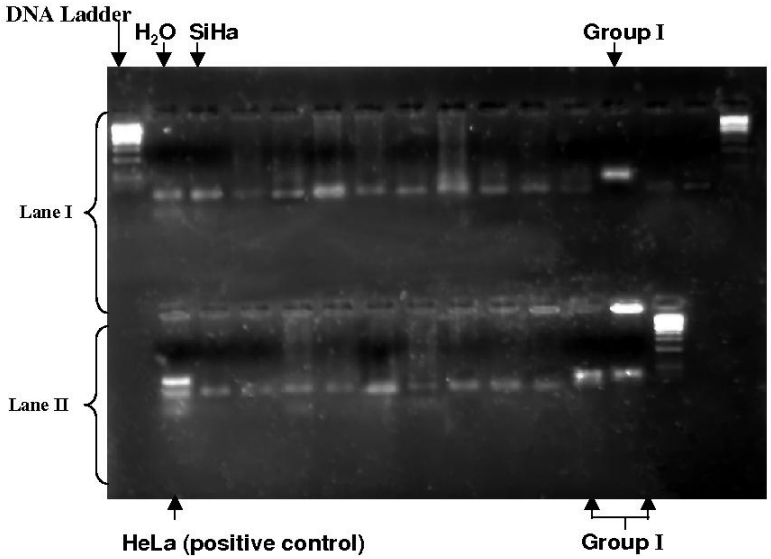
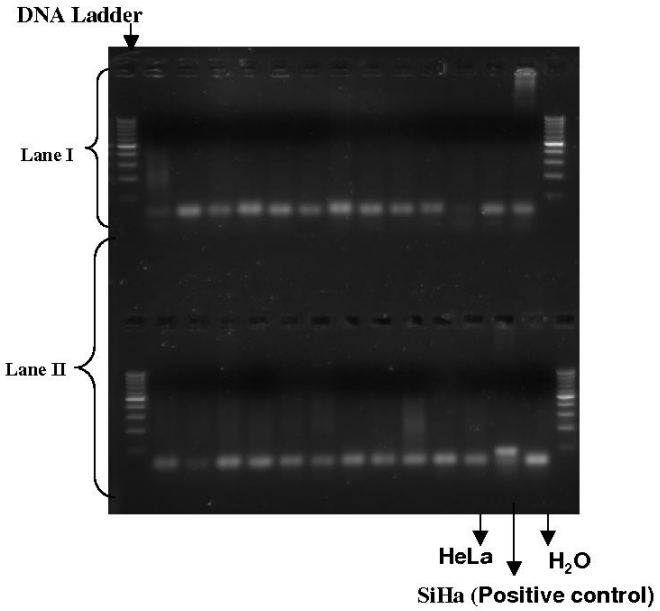


Figure 12: Gel showing Negative Amplification of HPV 16 in the Study Groups (n = 23)



Discussion

Viruses have been identified as etiological agents in several types of human cancers. High-risk types of HPVs (16 and 18) have been established as the cause of invasive cervical cancer and anogenital carcinomas. An increasing evidence implicating viruses as etiological agents in the development of cancers of the upper aerodigestive tract is present in the literature[19]. The role of HPV as an etiological agent in oral cancer is supported by the fact that HPV DNA is present in oral cancer tissue and the observation that high risk HPV virus can also immortalize and transform normal oral epithelial cells. In this background the present study was conducted to detect the presence of HPV type 16 and 18 in OSCC, potentially premalignant lesions and normal mucosa.

In this study the number of cases was fixed initially as 60 and the cases were selected after the histopathological diagnosis. Only 23 samples were used for the study as the housekeeping gene could be amplified only in these samples. The reason for this could be that, PCR inhibitors e.g. proteinase K, detergents (used for DNA extraction) may prevent the PCR amplification by inhibiting the activity of the thermostable enzymes[14]. The change in the DNA integrity is due to the cross linking with proteins induced by formaldehyde[71]. Thus this limitation should be considered when PCR technique is used in archival tissue. Consequently, to make DNA extraction possible it has been suggested that primers of amplicon size lesser than 200 base pair should be used [3]. Mork J et al (2001)[58] in their study were able to extract DNA only from 160 of 228 cancer specimens which were formalin fixed and paraffin embedded.

14 cases in Group I, 5 cases in Group II and 4 cases in Group III yielded satisfactory DNA content for PCR.

In Group I the mean age was 58.4, in Group II it was 53 and in Group III it was 36.8. In Group I and Group III, the male to female ratio was 1:1. In Group II all were males.

In Group I the predominant site was buccal mucosa (57.1%) and this finding was consistent with the report of Siriwardena B et al (2006)[74]. In Group III 75% percent of the cases were from the buccal mucosa and 25% from the gingiva. Markette H et al (2005)[50] detected HPV in 26% of gingival biopsies and stated that gingiva could act as a possible reservoir of the virus. D'Costa J et al (1998)[17] suggested that the presence of HPV in normal oral mucosa could be due to a latent infection.

In Group I (100%) and Group II (100%) all the cases had the habit of tobacco usage, some cases also consumed alcohol. Thavarajah R et al (2006)[87] in their study of oral lesions in 500 psychoactive substance users, found that those who habitually used tobacco, alcohol and arecanut had a 20 fold higher risk of developing leukoplakia when compared to those who did not engage in these habits, in the Indian context.

The Qiagen spin column based DNA extraction kit was used in our study for DNA extraction from sections of paraffin wax embedded tissue and the DNA so obtained was subjected to the polymerase chain reaction (PCR) for detection of house keeping gene and HPV DNA. Initially the house keeping gene primers, β – globin (220 bp size) and 18 S genes (300 bp) were tried. However, due to the lack of DNA integrity the efficiency of the PCR based amplifications of the house keeping genes was not effective for primers greater than 200 bp products. Similar findings have been reported by Baay M et al (1996)[3] who suggested that when lesser than or equal to 200 bp primers of amplicon product size were used for paraffin embedded samples

amplification was seen. Glutamate Dehydrogenase primers were then chosen as the housekeeping gene, as the amplicon product size was 88 bp.

Out of these 60 cases, the 23 samples for which housekeeping gene was positive by PCR were selected for the identification of HPV type 16 and 18.

In the present study 3 cases (21%) of Group I, were positive for HPV 18, whereas none of the Group II and Group III cases were positive for either HPV 18 or HPV 16.

Our results were comparable with that of Boy S et al (2006)[7] who detected the presence of only HPV 18 by real time PCR in 7 cases of the 59 cases of OSCC. The site of the tumor, age and gender had no relation to the presence of HPV. Lucia G et al (2002)[46] in their study of 121 cases of OSCC and other mucosal lesions in an Italian population detected HPV 18 in 85%, of their cases and in contrast had a very low prevalence of HPV 16, 31 and 33. Balaram P et al (1995)[4] reported HPV type 18 in 47% and HPV 16 in 42% of 91 cases of OSCC in their study in the South Indian population unlike D' Costa J et al (1998)[17] who did not detect HPV type 18 in their study of OSCC, premalignant lesions and normal oral mucosa. In our study, HPV type 18 was detected in 3 cases of OSCC.

HPV 16 was not detected in any of the groups in our study. In contrast to our present study, D'Costa J et al (1998)[17] in a North Indian population detected, only HPV 16 but not HPV 18 in oral cancers and potentially malignant lesions. The authors compared presence of HPV in the tissue sections of OSCC, potentially malignant lesions and normal appearing oral mucosa. They observed that, in 15 cases (15%) of OSCC, 27 cases (34%) of potentially malignant lesions and 15 cases (31%) of the corresponding normal mucosa the subtype HPV 16 only was present. No

correlation was observed in their study between the presence of HPV and gender, age and tumor stage and differentiation.

We could probably explain the absence of HPV 16 in our study due to the geographical variation as documented in other studies among different populations [84, 88, 46].

Among those cases, which were positive for HPV 18, two were well differentiated carcinomas and one was moderately differentiated carcinoma. This finding was similar to that of Elamin F et al (1998)[21] who did not find an association between the presence of HPV and the grade of the tumor.

In the HPV positive cases, 2 were female patients and one case was a male patient. Cruz I et al (1996)[15] found a higher HPV prevalence in males than females in their study of 35 cases of OSCC.

In our study group all the cases in which HPV was detected had the habit of chewing and smoking of tobacco. This correlation with tobacco and HPV detection has been reported by Elaine M et al (2004)[22] in their study of 201 patients with head and neck cancers they found that tobacco had an additive effect and that alcohol consumption had a synergistic effect with HPV positive cancers. In contrast, Herrero R et al (2003)[34] reported lower HPV prevalence in tobacco chewers and smokers and Chen P et al (2002)[11] did not find any association between HPV and tobacco associated habits. They concluded that both HPV and the habits acted as independent factors in the pathogenesis of cancer. In our study, all the HPV positive cases were habitual users of tobacco indicating a possible correlation with tobacco as seen by Elaine M et al (2004)[22].

In our study, HPV 18 was detected in cases within the age range of 45 to 58 with a mean age being 51 years. This finding is consistent with the observations by

Cruz I et al (1996)[15] were they stated that HPV was predominantly detected only in the age below 60 years. The authors hypothesized that combined effects of the habits and HPV infection may lead to the early occurrence of OSCC in this age group. Another possibility is that an infection by other high risk HPV subtypes such as 31, 33, 35 (i.e. other than HPV 16 and HPV 18) may account for those OSCC cases who were HPV negative and were below the age group of 60 years. Further studies would be required to explore this finding.

The absence of HPV in premalignant lesions in our study could be due to a small sample size or the absence of the role of HPV in these lesions or the presence of other types of HPV, which were not studied. The results of this study indicate that HPV could be an etiological agent in some OSCC in patients below the age of 60 years. Tobacco habits could also play a role along with HPV for the development of OSCC. This study also highlights that HPV induced cancers can be a separate subgroup of cancers, in those individuals who are lesser than 60 years of age. This could be due to the dual mutagenic effects of HPV and the combined effect of habits. In conclusion HPV 18 could be detected in 21% cases of oral cancer in this study, pointing to a possible role for HPV 18 in oral cancer. Further studies on a larger sample size could help to better understand the relation between HPV and oral cancer.

Summary & Conclusion

- 60 cases were selected. Good quality DNA for house keeping gene amplification could be obtained only for 23 cases.
- 23 cases were included in our study, divided into 3 groups.
- In Group I, 14 cases were histopathologically confirmed cases of OSCC of which 7 cases were well differentiated, 6 cases were moderately differentiated and 1 case was of poorly differentiated grade of OSCC.
- In Group II, 5 cases were histopathologically confirmed cases of epithelial dysplasia of which 3 cases had mild dysplasia and 2 cases had moderate dysplasia.
- In Group III, normal oral mucosal tissues were obtained from 4 cases.
- Male to female ratio was 1:1 in Group I and Group III. In Group II all were male patients.
- Mean age of cases in Group I was 58.4, Group II was 53 and in Group III mean age was 36.8.
- Tissues from the three groups were amplified for the identification of human papilloma virus type 16 and 18 using type specific primers by PCR method.
- HPV 18 was detected in 3 cases of Group I
- HPV18 was not detected in Group II and Group III
- HPV 16 was not detected in Group I, Group II and Group III

Our study indicates that HPV 18 has a probable role in the etiology of OSCC. However further studies in larger sample size will have to be done to establish this.

Bibliography

1. **Al Moustafa AE, Foulkes WD, Benlimame N, Wong A, Yen L**
   E6/E7 proteins of HPV type 16 and ErbB-2 cooperate to induce neoplastic transformation of primary normal oral epithelial cells
   Oncogene. 2004; 23: 350–358.
2. **Al Moustafa AE, Foulkes WD, Wong A, Jallal H, Batist G et al**
   Cyclin D1 is essential for neoplastic transformation induced by both E6/E7 and E6/E7/ErbB-2 cooperation in normal cells
   Oncogene. 2004; 23: 5252–5256.
3. **Baay MF, Quint WG, Koudstaal J, Hollema H, Duk JM, Burger MP**
   Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas.
   J. Clin. Microbiol 1996; 34:745–747.
4. **Balaram P, Nalinakumari KR., Abraham E, Balan A, Hareendran NK, Bernard HU, and Chan SY**
   Human papillomaviruses in 91 oral cancers from Indian betel quid chewers-high prevalence and multiplicity of infections.
   Int. J. Cancer. 1995; 61: 450–454.
5. **Bodaghi S, Wood LV, Roby G, Ryder C, Steinberg SM, Zheng ZM**
   Could human papillomaviruses be spread through blood
   Journal of Clinical Microbiology. 2005; 43:5428-34
6. **Bousarghin L, Touze A, Sizaret PY, Coursaget P**
   Human papillomavirus Types 16, 31, and 58 use different endocytosis pathways to enter cells.
   Journal of Virology. 2003 Mar; 77:3846-50
7. **Boy S, Rensburg E, Engelbrecht S, Dreyer L, Heerden M, Heerden W**
   HPV detection in primary intra-oral squamous cell carcinomas – commensal, aetiological agent or contamination?
   J Oral Pathol Med. 2006 35: 86–90

8. **Braakhuis BJ, Snijders PJ, Keune WJ, Meijer CJ, Ruijter-Schippers HJ, Leemans CR**

   Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papilloma virus.

   J Natl Cancer Inst. 2004; 96:998-1006.

9. **Brown DR, Schroeder JM, Bryan JT, Stoler MH, Fife KH**

   Detection of multiple human papillomavirus types in condylomata acuminata lesions from otherwise healthy and immunosuppressed patients

   J Clin Microbiol. 1999; 37:3316-3322

10. **Burger RA, Monk BJ, Kurosaki T, Anton-Culver H, Vasilev SA, Berman ML, Wilczynski SP**

    HPV type 18:Association with poor prognosis in early stage cervical cancer

    J Natl Cancer Inst. 1996; 88:1361-1368.

11. **Chen PC, Pan CC, Kuo C, Lin CP**

    Risk of oral cancer associated with HPV infection, betel quid chewing and cigarette smoking in Taiwan.

    J Oral Path Med. 2002; 31:317

12. **Chen SL, Lin ST, Tsai TC, Hsiao WC, Tsao YP**

    ErbB4 (JM-b/CYT-1)-induced expression and phosphorylation of c-Jun is abrogated by human papillomavirus type 16 E5 protein

    Oncogene 2006, 1–12

13. **Cinzia M, Pawel GF, Fabrizio G**

    Evidence for the association of human papilloma virus infection and cutaneous squamous cell carcinoma in immunocompetent Individuals.

    Arch Dermatol 2003; 139:890-894

14. **Crocker J and Burnett D**

    Science of laboratory diagnosis.

    Isis Medical Media Ltd 1998, Ed1, 234

15. **Cruz IBF, Snijders PJF, Steenbergen RDM, Meijer CJLM, Snow GB, Walboomers JMM and van der Waa1 I**

    Age-dependence of human papillomavirus DNA presence in oral squamous cell carcinomas

    Oral Oncol. 1996; 32B: 55-62

16. **Day PM, Baker CC, Lowy DR, Schiller JT**

Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression

PNAS. 2004; 101:14252–14257

17. **D'Costa J, Saranath D, Dedhiaa P, Sanghvi V, Mehtab A**

Detection of HPV-16 genome in human oral cancers and potentially malignant lesions from India

Oral Oncology.1998; 34:413-420

18. **De Villers, Neumann C, Le JY, Weidauer, zur Hausen**

Infection of the oral mucosa with defined types of human papillomaviruses.

Med Microbiol Immuno Berl. 1986;174:287

19. **Devita V T, Hellman S, Rosenberg S A**

Principles and Practice of oncology.

Lippincott 2001; 1st Ed. 124-126

20. **Durst M, Gissmann L, Ikenberg H, zur Hausen**

A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions.

PNAS. 1983; 80:3812

21. **Elamin F, Steingrimsdottir H, Wanakulasuriya S, Johnson N, Tavassoli M**

Prevalence of human papillomavirus infection in premalignant and malignant lesions of the oral cavity in U.K subjects: a novel detection method

Oral Oncology 1998; 34:191-197

22. **Elaine M S, Justine M R**

Human papillomavirus in oral exfoliated cells and risk of head and neck cancer

J Natl Cancer Inst 2004; 96: 449-55.

23. **Fan X, Liu Y, Chen JJ**

Down-regulation of p21 contributes to apoptosis induced by HPV E6 in human mammary epithelial cells

Apoptosis 2005; 10: 63–73

24. **Ferber MJ, Thorland EC, Brink AA, Rapp AK, Phillips LA, McGovern R**

Preferential integration of human papillomavirus type 18 near the c-myc locus in cervical carcinoma.

Oncogene. 2003; 22: 7233–7242

25. **Flint S J , Racaniello, Enquit L W, Skalka A M, Krug R M**

Virology - Molecular Biology, Pathogenesis and Control

ASM 2000; Ed 1: pg10

26. **Florin L, Sapp C, Streeck RE and Sapp M**

Assembly and translocation of papillomavirus capsid proteins.

Journal of Virology.2002; 76: 10009

27. **Flores ER, Allen-Hoffmann BL, Lee D, Lambert PF**

The human papillomavirus Type 16 E7 oncogene is required for the productive stage of the viral life cycle

Journal of Virology. 2000; 74: 6622-6631

28. **Genther Williams SM, Disbrow GL, Schlegel R, Lee D, Threadgill DW, Lambert PF**

Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a High-Risk human papillomavirus oncogene

Cancer Research. 2005; 65: pg 6534-6542

29. **Gissmann and zur Hausen**

Molecular cloning and characterization of human papillomavirus DNA derived from a larygeal papilloma .

Journal of Virology. 1982; 44:393

30. **Gillison L**

HPV-Associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity.

Semin Oncol. 2004;31: 744

31. **Glanna B, Aldo V and Armando B**

Overexpression of p53 and Bcl2 proteins and the presence of HPV infection are independent events in head and neck cancer.

J.Oral Path Med. 2000; 29:173

32. Haraf DJ, Nodzenski E, Brachman D, Mick R, Montag A, Graves D

HPV and p53 in Head and Neck Cancer: clinical correlates and survival.

Clinical Cancer Research. 1996; 2: 755

33. Helt A M and Galloway D

Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins

Carcinogenesis. 2003; 2:159-169

34. Herrero R, Castellsague X, Pawlita M, Lissowska J, Kee F, Balaram P

Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study.: International Agency for research on cancer Multicenter study.

J Natl Cancer Inst. 2003; 95:1772-1783

35. IACR (International agency on cancer research)

Human papillomaviruses (HPV) Data Reported and Evaluation.

IACR. 1995; 64: 1-6

36. Ibieta BR, Lizano M, Fras-Mendivil M, Barrera JL, Carrillo A, Ma Ruz-Godoy L

Human papilloma virus in oral squamous cell carcinoma in a Mexican population

Oral Surg, Oral Med, Oral Path, Oral Radiol Endo 2005; 99:311-5

37. Jayasooriya P R, Abeyratne S, Ranasinghe A W, Tilkarantne WM

Focal epithelial hyperplasia (heck's disease) report with PCR detection of human papillomavirus

Oral Dis. 2004; 10:240-243

38. Jimenez C, Correnti M, Salma N, Cavazza ME

Detection of human papillomavirus DNA in benign oral squamous epithelial lesions in Venezuela.

J Oral Pathol Med. 2001; 30:385-388

39. Johnson N W

Oral Cancer Detection of patients and lesions at risk. Risk markers for oral diseases

Cambridge Press; Ed 1, Pg 4-22

40. Jones DL, Alani RM, Munger K

The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2

Genes and Development 1997; 11: 2101-2111

41. Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T, Ishibashi M

Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein

PNAS. 1997; 94: 11612-11616

42. Kojima A, Maeda H, Sugita Y, Tanaka S, Kameyama Y

Human papillomavirus type 38 infection in oral squamous cell carcinomas.

Oral Oncol. 2002;38:591-596

43. Li W, Thompson CH, O'Brien CJ, McNeil EB, Scolyer RA, Cossart YE

Human papilloma virus positivity predicts favourable outcome for squamous carcinoma of the tonsil.

Int.J.Cancer. 2003 ;106:553

44. Liu JS, Kuo SR, Makhov AM, Cyr DM, Griffith JD, Broker TR

Human Hsp70 and Hsp40 Chaperone proteins facilitate human papillomavirus 11 E1 protein binding to the origin and stimulate cell-free DNA replication

J Biol Chem. 1998; 273: 30704-30712

45. Longworth MS, Laimins LA

Pathogenesis of human papillomaviruses in differentiating epithelia.

Microbiol Mol Biol Rev. 2004;68:362-372

46. Lucia G, Giuseppina , Anna L, Ornella G, John O, Valerio M

Human papillomavirus DNA in oral mucosal lesions.

The Journal of Infectious Diseases. 2002; 185: 833-836

47. Ma T, Zou N, Lin BY, Chow LT, Harper JW

Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication

PNAS. 1999; 96: 382-387

48. Maido R, Anu R, and Mart U

Human papilloma virus Type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism.

Journal of Virology. 1999; 73: p. 3062-3070

49. Majewski S, Jablonska S

Do epidermodysplasia verruciformis human papillomaviruses contribute to malignant and epidermal proliferation.

Arch Dermatol. 2002; 138: 649-654

50. Markette H, Jaana W, Hellevi R

Marginal periodontium as a potential reservoir of human papillomavirus in oral mucosa.

J Periodontol. 2005; 76:358-363

51. Martin LG, Demers GW, Galloway DA

Disruption of the G1/S transition in human papillomavirus type 16 E7-expressing human cells is associated with altered regulation of Cyclin E.

Journal of Virology. 1998;72: 975-985

52. Masterson PJ, Stanley MA, Lewis AP, Romanos MA

A C-Terminal helicase domain of the human papillomavirus E1 protein binds E2 and the DNA Polymerase -Primase p68 Subunit

Journal of Virology. 1998; 72: 7407-7419

53. Meisels A and Fortin R

Condylomatous lesions of the cervix and vagina.

Int. Cytological Pattern.Acta Cyto. 1976; 20:505

54. Michael D

Oral sex linked to mouth cancer.

http://www.newscientist.com/article.ns

55. Miller CS, Zeuss MS, White DK

Detection of HPV DNA in oral carcinoma using polymerase chain reaction together with in- situ hybridization

Oral Surg, Oral Med, Oral Path, Oral Radiol Endo 1994; 77:480-486

56. Miller CS, White DK

HPV expression in oral mucosa, premalignant conditions and squamous cell carcinoma

Oral Surg, Oral Med, Oral Path, Oral Radiol Endo 1996; 82:57-68

57. Miller CS, Johnstone BM

Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982-1997.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001 Jun;91:622-635

58. Mork J, Lie AK, Glattre E, Hallmans G, Jellum E, Koskela P

Human papilloma virus infection as a risk factor for squamous cell carcinoma of the head and neck

N Engl J. Med 2001; 344: 1125-1131

59. Neal N, Ahmed R, Scrano F G

Viral pathogenesis

Lippincott: 1(1), 268

60. Neville, Damm, Allen, Bouquot

Oral and Maxillofacial Pathology

Elsevier. 2nd ed, 2002, 337

61. Nguyen DX, Westbrook TF, McCance DJ

Human papillomavirus Type 16 E7 maintains elevated levels of the cdc25A Tyrosine Phosphatase during deregulation of cell cycle arrest.

Journal of Virology. 2002; 76: 619-632

62. Oishee C, Karthikeyan V, Vinay T

Human Papillomavirus Type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras

Journal of Virology. 2004; 78: 5934–5945

63. Okun MM, Day PM, Greenstone HL, Booy FP, Lowy DR, Schiller JT, Roden RB

L1 interaction domains of papillomavirus L2 necessary for viral genome encapsidation

Journal of Virology. 2001; 75: 4332–4342

64. Patel D, Huang SM, Baglia LA, McCance DJ

The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300

The EMBO Journal. 1999; 18: 5061-5072

65. Pinto J, Franco E L, Jean B, Arella M

Human papillomaviruses and prognosis of patient with cancers of upper aerodigestive tract

Cancer. 1999; 85:1903

66. Pradip N, Kumaravel S, and Sudhir K

Activated Notch1 Inhibits p53-Induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent Pathway

Journal of Virology. 2003; 77: 7106–7112

67. Roberto E V M, Luisia L V, Anoi CC

Low prevalence of human papilloma virus in geographic regions with high incidence of Head and Neck Cancers

AM J Surg 1998; 176: 428-429

68. Schreiber K, Cannon RE, Karrison T, Beck-Engeser G, Huo D, Tennant RW

Strong synergy between mutant ras and HPV16 E6/E7 in the development of primary tumors

Oncogene 2004; 23: 3972–3979

69. Seth R S, Bevan Y, James K M, Janet K M, Stephen M S

Human papillomaviruses infection and survival in Oral Squamous Cell Carcinomas

Otolaryngol Head and Neck Surg 2001; 125: 1-9

70. Shafti-Keramat S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer

Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses

Journal of Virology 2003; 77: 13125-13135

71. Shedlock M A, Haygood G M, Pietsch TW

Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin fixed museum specimens.

Biotechniques. 1997; 22: 394-400

72. Shima K, Kobayashi I, Saito I, Kiyoshima T, Matsuo K, Ozeki S, Ohishi M

Incidence of HPV 16 &18 infection and mutation of p53 in patients with oral squamous cell carcinoma in Japan

Br. J. Oral Surg 2000; 38:445

73. Shope RE, Hurst EW

Infectoius papillomatosis of rabbits. With a note on histopathology.

J Exp Med. 1933; 58:607

74. Siriwardena BSMS, Tilakaratne A, Amaratunga EAPD, Tilakaratne WD

Demographic, etiological and survival difference of oral squamous cell carcinoma in the young and old in Srilanka

Oral Oncology 2006; 42:831-836

75. Stewart D, Ghosh A, Matlashewski G

Involvement of nuclear export in human papillomavirus type 18 E6-mediated ubiquitination and degradation of p53

Journal of Virology. 2005; 79: 8773–8783.

76. Strauss MJ, Shaw EW, Bunting H, Melnick L

Crystalline virus like particle from skin papillomas characterized by intra inclusion bodies.

Proc Soc Exp Biol Med. 1949; 72: 46

77. Subramanyam D, Krishna S

c-Myc substitutes for Notch1-CBF1 functions in cooperative transformation with papillomavirus oncogenes

Virology 2006; 347:191–198

78. Sugiyama M, Bhawal UK, Dohmen T, Ono S, Miyauchi M

Detection of human papillomavirus-16 and HPV-18 DNA in normal,dysplastic and malignant oral epithelium.

Oral Surg, Oral Med, Oral Path, Oral Radiol Endo 2003; 95:594-600

79. Susanne I W, Delicia A F, Alla Y K Jennifer J DJohn D B and Peter M H
Papillomavirus E2 induces senescence in HPV-positive cells via pRB and p21CIP -dependent pathways.
EMBO Journal. 2000; 19: 5762-5771

80. Swindle CS, Engler JA
Association of the human papillomavirus Type 11 E1 protein with histone H1
Journal of Virology. 1998;72: 1994-2001

81. Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuutinen J
Morphological and immunohistochemical evidence suggesting human papilloma virus (HPV) involvement in oral squamous cell carcinogenesis.
Int .J. Oral Surg.1983; 12:418-424

82. Syrjanen S and Puronen M
Human papillomaviruses infections in children: the potential role of maternal transmission
Crit Rev Oral Biol Med 2000; 11: 259-274

83. Tachezy R, Klozar J and Salakova M Smith E, Turek L, Betka J
HPV and other risk factors of oral cavity /oropharyngeal cancer in Czech republic.
Oral Diseases. 2005; 11:181-185

84. Tang X, Jia L, Ouyang J, Takagi M
Comparative study of human papilloma virus prevalence in Japanese and North-East Chinese oral carcinoma
J.Oral Path Med 2003; 32:393-398

85. Terai M, Hashimoto K, Yoda K, Sata T
High prevalence of human papilloma viruses in the normal oral cavity of adults
Oral Microbiol Immunol 1999; 14: 201–205

86. Terai M, Takagi M, Matsukura T
Oral wart associated with HPV type 2.
J Oral Pathol Med. 1999; 28: 137-140

87. Thavarajah R, Rao A, Raman U, Rajasekaran S, Joshua E, Kannan R
Oral lesions of 500 habitual psychoactive substance users in Chennai, India.
Archives of Oral Biology. 2006; 51: 512-519

88. Tsuhako K, Nakazato I, Miyagi J, Iwamasa T, Arasaki A, Hiratsuka H
Comparative study of oral squamous cell carcinoma in Okinawa, Southern Japan and Sapporo in Hokkaido, Northern Japan; with special reference to human papillomavirus and Epstein-Barr virus infection
J.Oral Path Med 2000; 29:70

89. Veldman T, Liu X, Yuan H, Schlegel R
Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter.
PNAS. 2003; 100: 8211-8216

90. Von Knebel D M, Oltersdorf T, Schwarz E, Gissmann L
Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV 18 E6-E7 antisense RNA.
Int J Cancer. 1992; 51:831

91. Yang R, Day PM, Yutzy W, Lin KY,Hung CF and. Roden RBS
Cell surface-Binding motifs of L2 that facilitate papillomavirus infection.
Journal of Virology. 2003: 77; 3531–3541

92. Yang R, Yutzy WH, Viscidi RP, Roden RB
Interaction of L2 with β-Actin directs intracellular transport of papillomavirus and infection
J of Biological Chemistry. 2003; 278: 12546–12553

93. Yuan-Hong L, Xing-Hua G, Chin-Di H
Detection of HPV and response to oral arotiniods Ethylester in 2 cases of Darier's diseases
Arch Dermatol.2002; 138: 695-696

94. Zhang ZY, Sdeck P, Cao J,Chen WT
Human papillomavirus type 16 and 18 DNA in oral squamous cell carcinoma and normal mucosa
Int J Oral Maxillofac Surg. 2004; 33: 71-74

95. zur Hausen H

Papilloma viruses and cancer: From basic studies to clinical application

Nature. 2002; 2: 342-349

Annexure

HPV Types and Diseases

HPV Type	Location	Association
1	Cutaneous	Plantar warts
2	Cutaneous	Common warts
3	Cutaneous	Flat warts
4	Cutaneous	Common and plantar Warts
5	Genital tract, other mucosae	Macular lesions in EV and cancers
6	Genital tract, other mucosae	Genital Warts, laryngeal papillomatosis
7	Cutaneous	Butcher's warts
8	Cutaneous	Macular lesions (EV) and cancers
9	Cutaneous	Macular lesions (EV)
10	Cutaneous	Flat warts
11	Genital tract, other mucosae	Genital warts, laryngeal papillomas
12	Cutaneous	Macular lesions (EV)
13	Oral	Oral focal epithelial hyperplasia
14	Cutaneous	Macular lesions and cancers
15	Cutaneous	Macular lesions
16	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
17	Cutaneous	Macular lesions (EV) and cancers
18	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
19	Cutaneous	Macular lesions (EV)
20	Cutaneous	Macular lesions (EV) and cancers
21	Cutaneous	Macular lesions (EV)
22	Cutaneous	Macular lesions (EV)
23	Cutaneous	Macular lesions (EV)
24	Cutaneous	Macular lesions (EV)
25	Cutaneous	Macular lesions (EV)
26	Cutaneous	Common warts
27	Cutaneous	Common warts
28	Cutaneous	Flat warts
29	Cutaneous	Common warts
30	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
31	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
32	Oral	Oral focal epithelial hyperplasia
33	Genital tract	Intraepithelial neoplasia and cancers
34	Genital tract, other mucosae	Intraepithelial neoplasia
35	Genital tract, other mucosae	Intraepithelial neoplasia and cancers

36	Cutaneous	Actinic keratosis, EV lesions
37	Cutaneous	Not yet known, isolated from a keratoacanthoma
38	Cutaneous	Not yet known, isolated from a melanoma
39	Genital tract, other mucosae	Intraepithelial neoplasia and cancer
40	Genital tract, other mucosae	Intraepithelial neoplasia and cancer
41	Cutaneous	Flat warts
42	Genital tract, other mucosae	Intraepithelial neoplasia
43	Genital tract, other mucosae	Intraepithelial neoplasia
44	Genital tract, other mucosae	Intraepithelial neoplasia
45	Genital tract, other mucosae	Intraepithelial neoplasia
46	Cutaneous	Macular lesions (EV)
47	Cutaneous	Macular lesions (EV)
48	Cutaneous	Cutaneous squamous cell carcinoma (Transplant patient)
49	Cutaneous	Flat Warts in immunocompromised patient
50	Cutaneous	Macular lesions (EV)
51	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
52	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
53	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
54	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
55	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
56	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
57	Oral, genital	Oral papillomas and inverted maxillary sinus papillomas
58	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
59	Genital tract, other mucosae	Anogenital intraepithelial neoplasia
60	Cutaneous	Epidermoid Cysts, plantar warts
61	Genital tract, other mucosae	Intraepithelial neoplasia
62	Genital tract, other mucosae	Intraepithelial neoplasia

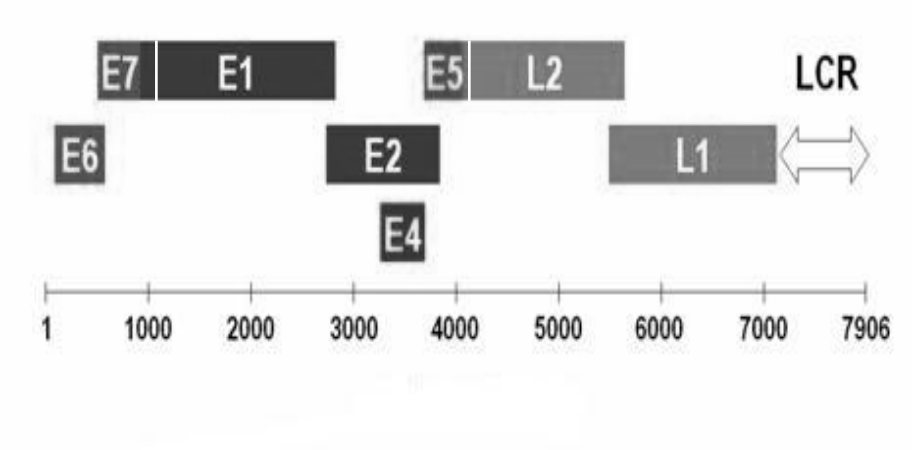
63	Cutaneous	Isolated from a plantar wart
64	Genital tract, other mucosae	Intraepithelial neoplasia
65	Cutaneous	Isolated from a pigmented wart
66	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
67	Genital tract, other mucosae	Isolated from an intraepithelial neoplasia
68	Genital tract, other mucosae	Isolated from an intraepithelial neoplasia
69	Genital tract, other mucosae	Intraepithelial neoplasia and cancers
70	Genital tract, other mucosae	Isolated from a vulvar papilloma
71	Genital tract, other mucosae	Isolated from an intraepithelial neoplasia,
72	Oral	Isolated from an oral papilloma (HIV patient)
73	Oral	Isolated from an oral papilloma (HIV patient)
74	Genital tract, other mucosae	Isolated from an intraepithelial neoplasia,
75	Cutaneous	Isolated from a common wart in organ allograft recipient
76	Cutaneous	Isolated from a common wart in organ allograft recipient
77	Cutaneous	Isolated from a common wart in organ allograft recipient

EV - Epidermodysplasia Verruciformis

Oral HPV Types and Diseases

Genotype	Diseases
HPV 2, 4	Verruca Vulgaris
HPV 6, 11	Condyloma Acuminatum, Squamous Papilloma
HPV 13, 32	Focal Epithelial Hyperplasia
HPV 16	Proliferative Verrucous Leukoplakia
HPV 6, 11, 16	Verrucous Carcinoma
HPV 16, 18	Squamous Cell Carcinoma

HPV Genome


E7
E1
E5
L2
LCR
E6
E2
L1
E4
1
1000
2000
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4000
5000
6000
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